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STUDIES ON THE AQUEOUS HUMOUR

HUGH DAVSON AND CHARLES BEECHER WELD

From the Department of Physiology, Dalhousie University, Halifax, N. S.

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The aqueous humour may be considered to be *a*, an ultra-filtrate of plasma in thermodynamic equilibrium with it; *b*, a secretion of the cells of the epithelial lining of the eye, or *c*, a fluid which owes its origin to an initial filtration process, but whose composition is modified by the secretion into, or the absorption from it of certain constituents. The experimental evidence in relation to these theories has been discussed at length by Davson and Quilliam (1940) and by Duke-Elder, Quilliam and Davson (1940) and it was suggested that the last named hypothesis was most consistent with the facts, especially in view of the divergence of the experimentally determined distribution of chloride between the aqueous humour and plasma (Hodgson, 1938) and of the osmotic pressures of the two fluids (Benham, Duke-Elder and Hodgson, 1938; Roepke and Hetherington, 1940) from those required on the basis of thermodynamic equilibrium. This equilibrium demands a distribution ratio, $R_{Cl} = [Cl]_{Serum}/[Cl]_{Aqueous}$ equal to 0.96 (Van Slyke, 1926) whereas Hodgson reported an average value for dogs of 0.92[1], and it might be thought that sodium chloride is being secreted into the aqueous humour so that the chloride concentration is greater in this fluid than the equilibrium demands. In this case the distribution of sodium should also be divergent from theory; however Davson (1939) has shown by methods accurate to within 1 in 500 that in the cat the mean distribution ratio, $R_{Na} = [Na]_{Serum}/[Na]_{Aqueous}$ is 1.03, i.e., close to the theoretical one of 1.04, and that the mean chloride distribution ratio was 0.945, and therefore not so divergent as in the dog.

In the present work the distribution of sodium has been examined in the dog (a more suitable experimental animal than the cat since it is possible to remove eye fluids with only local anesthesia); further, the distribu-

[1] Hodgson does not report the protein contents of his sera, so that we have calculated this ratio on the assumption of a mean value of 7 per cent.

tion of chloride has been investigated under several experimental conditions, and finally the ability, or otherwise, of the eye membranes to exclude inulin, injected into the blood, from penetrating the eye fluid has been tested. These last experiments were carried out to see whether it is likely that these membranes can have any effective secretory activity. Inulin has a molecular weight of 5000, and being a polysaccharide has a large number of water-soluble groups in it, and may consequently be expected to diffuse through a membrane, if at all, by way of the inter-cellular spaces. If these spaces are large enough to permit its passage through them, it is very unlikely that the cells constituting the epithelial lining of the eye would be able to secrete substances into the eye at rates great enough to maintain appreciable concentration gradients over a period of time, owing to the rapidity with which the secreted substances would escape back to the blood stream through these large inter-cellular spaces.[2]

EXPERIMENTAL. In the studies on sodium distribution the aqueous humour was drawn from the cocainised eye without other anesthesia; the blood was drawn by puncture of the femoral vein. During the studies on the chloride distribution and inulin penetration it was found that essentially similar results were obtained with and without general anesthesia, so generally the dog was given sodium amytal intravenously. In the studies on the penetration of inulin, the concentration of this substance in the blood was raised to about 100 mgm. per cent by a single venous injection of 10 per cent inulin in isotonic NaCl, and then maintained at about that level by continuous intravenous injection with the same solution. The chemical methods for the determination of sodium and chloride were those described earlier (Davson, 1939); inulin was determined by estimating the total reducing value by the Hagedorn-Jensen (1923) method before and after hydrolysis with 0.1 N H_2SO_4. Excellent reproducibility (within the titration error of 2 per cent) was obtained by this method on samples of 0.2 ml. of fluid.

RESULTS. *Distribution of sodium.* In table 1 the concentrations of sodium, expressed in millimoles per kilogram H_2O, in the serum and aqueous humour of ten separate dogs are shown, giving a mean value of R_{Na} of 1.04, in excellent agreement with the theoretical value deduced by Van Slyke (1926) on the basis of an average base-binding power of plasma proteins. We may conclude from these results that the distribution of chloride, described by Hodgson, is not due to the secretion of extra sodium chloride into a filtrate of blood plasma, since if this occurred the value of R_{Na} would be 1.00 or less.

Distribution of chloride. Preliminary experiments confirmed Hodgson's observation that the value of R_{Cl} was less than 0.96 (the mean of 21 of

[2] We are indebted to Prof. F. R. Winton of University College, London, for suggesting this mode of approach.

the experiments carried out during this work gave a value of 0.93), and a variety of experiments have been performed to determine whether the result is an artifact of the experimental procedure; the detailed description of many of the results would be tedious, so some will be merely indicated.

a. The discrepant ratio is not due to the instillation of cocaine hydrochloride into the eye, since no difference in the concentrations of chloride in the aqueous humours of the two eyes of a dog was observed when only one eye was instilled with cocaine.

b. The discrepant ratio is not due to transient variations in the blood chloride, since experiments in which the blood chloride was followed for several hours before the removal of the aqueous humour showed only small

TABLE 1

Comparison of the concentrations of sodium in blood serum and aqueous humour of dogs

EXPERIMENT	SERUM	AQUEOUS HUMOUR	PER CENT SOLIDS IN SERUM	SERUM	AQUEOUS HUMOUR	R_{Na}
	millimols/kgm.	*millimols/kgm.*		*millimols/kgm. H_2O*	*millimols/kgm. H_2O*	
1	140.2	145.9	8.5	153.1	147.3	1.040
2	141.4	144.1	6.9	151.8	145.5	1.045
3	134.1	136.5	6.4	143.2	137.8	1.040
4	141.4	145.7	9.0	155.3	147.2	1.055
5	139.5	147.6	8.0	151.7	149.1	1.020
6	139.7	144.7	9.0	153.6	146.2	1.050
7	137.4	140.7	6.5	147.0	142.1	1.035
8	137.7	142.5	8.9	151.1	144.0	1.050
9	139.7	144.5	7.4	150.9	146.0	1.035
10	139.1	144.1	8.6	152.3	145.5	1.045
Mean						1.040
S.D.						0.01
S.E.						0.003

variations, and these showed no definite trend from one experiment to another.

c. The discrepant ratio is not due to the metabolic activity of the lens, since the removal of this body from the eye and subsequent determination of the chloride ratios in the two eyes separately gave, with some animals, identical chloride ratios in the two eyes The results of these experiments, in which the chloride ratios were followed as long as three months after the removal of the lens, are shown in table 2, and it may be seen that in experiment 1, after three months the concentration of chloride was the same in both eyes; similarly in experiment 2 after two months. In general the impression was gained that during the first month the concentration of chloride in the aphakic eye was less than in the normal (expts. 1, 2 and 3) but that the concentrations tended to become equal later.

The effect of paracentesis. After removal of the aqueous humour the eye refills rapidly, so that within 15 minutes sufficient of the new fluid can be drawn out and subjected to analysis. This fluid, unlike the normal aqueous humour, contains appreciable amounts of protein (a maximal concentration of 2 per cent was observed) which, however, soon disappears, so that within 8 hours the fluid is quite clear. If the aqueous humour is formed initially as a filtrate from plasma, and only subsequently subjected to secretory activity, the fluid reformed immediately after paracentesis may be expected to have a value of R_{Cl} characteristic of a filtrate of plasma, whilst the fluid withdrawn later, say within 12 hours, may be expected to have resumed its characteristic distribution ratio. In the following

TABLE 2

The effect of removal of the lens on the distribution of chloride between the blood and aqueous humour

EXPERIMENT NUMBER	FLUID	TIME AFTER REMOVAL OF LENS		
		1 month	2 months	3 months
		Concentration of chloride (mmols./kgm. H_2O)		
1	Normal aqueous	128.5	127.5	127.5
	Aphakic aqueous	127.0	126.0	127.5
	Serum	123.0	118.5	120.0
2	Normal aqueous	131.5	122.5	129.5
	Aphakic aqueous	127.0	125.5	131.0
	Serum	120.5	120.5	122.5
3	Normal aqueous	129.5	126.0	
	Aphakic aqueous	126.5	126.0	
	Serum	124.5	119.0	
4	Normal aqueous		127.5	
	Aphakic aqueous		129.0	
	Serum		119.0	

experiments aqueous humour was withdrawn from one eye, and at periods varying from 15 minutes to 24 hours the refilled aqueous humour and the normal humour from the other eye were withdrawn, together with a sample of the blood. The concentrations of chloride in the two fluids are shown in table 3, where the values of R_{Cl} for each eye are also shown. The experiments were all carried out on separate dogs. The table shows that within 15 minutes the value of R_{Cl} for the refilled eye is considerably greater than that for the normal eye (0.96 in comparison with 0.925); within eight hours the concentration of chloride in the two eyes is equal.[3]

[3] It may be argued that the presence of protein in the aqueous humour *per se* would increase the value of R_{Cl}; however it is unlikely that it would produce the large effects observed, and it is found that the effect on the sodium ratio is small.

Permeability to inulin. In these experiments the concentration of inulin in the blood was maintained at around 100 mgm. per cent for several hours and the aqueous humour was withdrawn from the two eyes at different intervals. In table 4 the results of a typical experiment are shown. (Normal plasma and aqueous humour invariably show an increase in the

TABLE 3

The effect of paracentesis on the distribution of chloride between the blood and aqueous humour of dogs

TIME AFTER PARACENTESIS	EYE	AQUEOUS HUMOUR CHLORIDE CONCN.	SERUM CHLORIDE CONCN.	R_{Cl}
		mmols./kgm. H_2O	*mmols./kgm. H_2O*	
15 minutes	Normal	126.0	116.5	0.925
	Refilled	121.5		0.960
2 hours	Normal	128.5	118.5	0.920
	Refilled	126.0		0.940
3 hours	Normal	126.0	118.5	0.940
	Refilled	124.0		0.955
5 hours	Normal	129.5	120.0	0.925
	Refilled	128.5		0.935
8 hours	Normal	127.0	115.4	0.910
	Refilled	127.0		0.910

TABLE 4

Changes in the reducing value, expressed as milligrams glucose per cent, of aqueous humour after injection of inulin into the dog

INTERVAL BETWEEN 1ST INJECTION OF INULIN AND WITHDRAWAL OF FLUID	FLUID	REDUCING VALUE BEFORE HYDROLYSIS	REDUCING VALUE AFTER HYDROLYSIS	REDUCING VALUE AFTER HYDROLYSIS CORRECTED	INULIN
3 hours	Left aqueous	95.5	105.5	98.5	3.0
6 hours	Right aqueous	91.5	102.0	95.0	3.5
30 minutes	Blood serum	117.5	260.0	253.0	135.5
3 hours	Blood serum	107.0	190.0	187.0	80.0
6 hours	Blood serum	107.0	228.0	221.0	114.0

reducing value after hydrolysis, generally of the order of 7 mgm. glucose per cent, and the values obtained have been corrected for this, as is shown in the table.) The table shows a small increase in the reducing value after hydrolysis in the aqueous humour, but since this is hardly greater in the six hour specimen than in the three hour one, it is unlikely that it can be ascribed to the penetration of inulin into the eye.

Discussion. The results on the distribution of chloride between the aqueous humour and serum suggest that the aqueous humour is not formed by passive filtration alone, because the ratio is too low, having a mean value in 21 experiments of 0.930 ± 0.006 (Standard Error). The rapid refilling of the eye after paracentesis, and the appearance of appreciable quantities of protein in the reformed fluid, suggest that in this case the reformed aqueous humour is simply a filtrate; such a view is supported by the observation that the chloride ratio in the fluid withdrawn 15 minutes after paracentesis is close to that required by the Donnan Equilibrium. The presence of protein in the reformed aqueous humour suggests that the rapid fall in intra-ocular pressure associated with paracentesis causes a stretching of the membranes separating the plasma from the eye fluids, increasing their inter-cellular spaces and thereby allowing some proteins to penetrate. (It may be mentioned here that inulin penetrates the eye rapidly after paracentesis.) In these circumstances it is to be expected that any secretory activity of the epithelium will be completely masked, owing to the large leaks in the inter-cellular spaces. With the repair of the leaks, which apparently occurs rapidly, the secretory activity may become effective and the chloride concentration attain its normal value in respect to that of plasma.

The fact that inulin does not penetrate the eye, although present in the plasma for as long as six hours, certainly suggests that one of the membranes, interposed between the capillary endothelium and the aqueous humour, is considerably more selective than the capillary endothelium itself, and this membrane is probably the epithelial lining of the vascularised structures of the eye, namely, the iris, ciliary body and choroid. The permeability of such complex membranes has been discussed by Chambers (1940) who has shown that their selectivity in regard to water soluble substances like sugars is largely determined by the packing of the individual cells and the nature of the inter-cellular cement. The impermeability of the eye membrane to inulin differentiates the latter from the capillary endothelium which is freely permeable to this substance. The further differentiation between the more closely packed intestinal epithelium type of membrane and the still more closely packed one characterised by the kidney tubular epithelium awaits further studies along the lines of the inulin experiments described here.

In conclusion we may state that although the bulk of the evidence regarding the nature of the aqueous humour suggests that it is formed simply as a passive filtrate from plasma, and consequently that the intra-ocular pressure will be determined largely by the simple physical factors of colloid osmotic pressure of the plasma and hydrostatic capillary pressure, other evidence suggests that super-imposed on this primary process of filtration there is a certain selective activity on the part of the cellular lining

of the eye. Whether or not this has any obvious functional significance in regard to the maintenance of the intra-ocular pressure cannot yet be decided; the fact that the extra chloride in the aqueous humour is not accompanied by extra sodium (potassium and calcium are also ruled out by the work of Davson, Duke-Elder and Benham, 1936, and of Stary and Winternitz, 1932) suggests that it is compensated by a deficiency of some other anion or alternatively that there is an excess of some organic cation in the aqueous humour.

SUMMARY

The distribution of sodium and chloride between the aqueous humour and blood plasma of dogs has been investigated under normal and experimental conditions and it has been concluded that although filtration represents the primary process in aqueous humour formation, super-imposed on this process there is evidence of some secretion. It has been shown that inulin cannot pass from the blood into the eye.

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THE EFFECT OF THE PITUITARY ADRENOCORTICOTROPIC HORMONE AND OF CORTICOSTERONE ACETATE ON INSULIN HYPOGLYCEMIA AND LIVER GLYCOGEN IN ADRENALECTOMIZED MICE

J. F. GRATTAN, H. JENSEN AND D. J. INGLE

From the Biochemical Laboratory of The Squibb Institute for Medical Research, New Brunswick, N. J., and the George S. Cox Medical Research Institute, University of Pennsylvania, Philadelphia

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It was first demonstrated by Houssay and Potick (1) and by Benedetto (2) that the administration of anterior pituitary extracts to normal or hypophysectomized animals inhibits the hypoglycemic action of subsequently injected insulin. These findings have since been substantiated by different investigators. Young (3, 4) has investigated the anti-insulin action of various anterior pituitary preparations and proposed the term "glycotropic" for this effect. He found that the pituitary substance which inhibits the hypoglycemic action of insulin was different from prolactin, the glycotropic factor, and the gonadotropic hormones. Jensen and Grattan (5) reported the results of studies in normal mice indicating that the anti-insulin[1] effect of the anterior pituitary may be attributed to the adrenocorticotropic principle of that gland and that the effect is mediated through the adrenal cortex. Grattan and Jensen (6) have demonstrated that apparently only those principles of the adrenal cortex substituted in ring 3 (keto- or hydroxy group) exert this effect. This finding is in agreement with the observations of various other investigators (7, 8, 9, 10), that apparently only those adrenal cortical principles, which contain either a keto- or hydroxy group at C_{11}, exert a significant influence on carbohydrate metabolism. Grattan and Jensen (6) suggested that the anti-insulin response produced by the adrenocorticotropic hormone and the corticosterone-like compounds is probably due to the ability of these substances to promote the formation of liver glycogen. Hartman and his associates (11) have likewise concluded that the increased resistance to insulin of normal fasted mice after cortin injections is apparently due to gluconeogenesis, since both the blood sugar and liver glycogen are elevated.

[1] The term "anti-insulin" as employed by us refers only to the ability of a substance to counteract the hypoglycemia subsequent upon the injection of insulin into an animal. One has to distinguish between specific and unspecific effects. The unspecific effects will be discussed in another paper.

The anti-insulin response elicited by certain adrenocortical principles in normal mice may be used as an index of the adequacy of their effect on carbohydrate metabolism.

The object of this study was to extend the observations on normal animals by determining the effect of the adrenocorticotropic pituitary factor and of corticosterone on insulin hypoglycemia and liver glycogen in adrenalectomized mice under similar experimental conditions as previously employed (5, 6).

TABLE 1

Anti-insulin tests in normal and adrenalectomized mice

The corticosterone acetate was administered in peanut oil, 0.2 cc. containing 0.5 mgm. of the steroid; the adrenocorticotropic preparation was injected in aqueous solution of pH = 7.5, 0.5 cc. containing 5.0 mgm. of the hormone, all injections subcutaneously.

PREPARATION INJECTED	CONDITION OF ANIMALS	INSULIN DOSE PER KILO	TOTAL NUMBER OF ANIMALS	NUMBER OF CONVULSIONS	PER CENT CONVULSIONS
		units			
Controls*	Intact	1.5 or 2.0	202	179	89
5.0 mgm. adrenocorticotropic*	Intact	1.5 or 2.0	128	21	16
0.5 mgm. corticosterone acetate*†	Intact	1.5	13	0	0
Controls	Adrenalectomized	0.4	33	30	90
5.0 mgm. adrenocorticotropic‡	Adrenalectomized	0.4	16	14	88
0.5 mgm. corticosterone acetate†	Adrenalectomized	0.4	16	2	13

* These values were taken from table 1 of a previous publication (6).

† Supplied by Dr. E. C. Kendall.

‡ Same preparation as used in previous experiments (5, 6).

EXPERIMENTAL. Male mice weighing from 20 to 25 grams were adrenalectomized approximately 6 days before the experiments. During the postoperative period, they were maintained on a daily subcutaneous injection of 0.1 mgm. desoxycorticosterone acetate in 0.1 cc. of peanut oil and received 0.9 per cent sodium chloride in their drinking water.

Anti-insulin test. The procedure followed in determining the anti-insulin effect of the two hormones was the same as that previously employed by Jensen and Grattan (5) except for the following changes: *1.* Due to the greater insulin-sensitivity of adrenalectomized mice, 0.4 unit of insulin per kilo was found to be sufficient to produce a high percentage of convulsions, this dose represents one-fifth of the insulin dose employed

in the studies in normal mice (2 units per kilo). *2.* Before the 6 hour test period, all animals received their daily maintenance dose of desoxycorticosterone acetate. *3.* Controls for the corticosterone tests received peanut oil. *4.* During the 6 hour fast, the animals had access to 0.9 per cent sodium chloride in their drinking water.

The anti-insulin response of the two substances in adrenalectomized mice is recorded in table 1.

TABLE 2

Effect of pituitary adrenocorticotropic hormone and of corticosterone acetate on liver glycogen of normal and adrenalectomized mice

The corticosterone acetate was administered in peanut oil, 0.2 cc. containing 0.5 mgm. of the steroid; the adrenocorticotropic preparation was injected in aqueous solution of pH = 7.5, 0.5 cc. containing 5.0 mgm. of the hormone, all injections subcutaneously.

PREPARATION INJECTED	NUMBER OF ANIMALS	CONDITION OF ANIMALS	AVERAGE WEIGHT OF ANIMAL AT ONSET OF FAST	AVERAGE WEIGHT LOSS PER ANIMAL DURING FAST	AVERAGE LIVER WEIGHT PER ANIMAL	LIVER GLYCOGEN
			grams	*grams*	*grams*	*mgm. per cent*
Controls*	72	Intact	19.9	0.90	1.18	616
5.0 mgm. adrenocorticotropic*	28	Intact	20.3	0.23	1.32	2,346
0.5 mgm. corticosterone acetate*†	28	Intact	19.7	1.07	1.19	2,127
Controls	21	Adrenalectomized	20.9	0.67	1.28	440
5.0 mgm. adrenocorticotropic‡	25	Adrenalectomized	21.5	0.21	1.34	305
0.5 mgm. corticosterone acetate†	12	Adrenalectomized	21.8	0.85	1.47	3,415

* These values were taken from table 2 of a previous publication (6).

† Supplied by Dr. E. C. Kendall.

‡ Same preparation as used in previous experiments (5, 6).

Effect on liver glycogen. It has been found by Grattan and Jensen (6) that the adrenocorticotropic hormone as well as corticosterone and chemically related compounds greatly increase the liver glycogen of normal mice during a six hour fast, while desoxycorticosterone failed to do so. Both adrenocorticotropic hormone and corticosterone acetate were tested for their effect on liver glycogen in adrenalectomized mice according to the procedure employed by Grattan and Jensen (6). The animals received the same preliminary treatment as in the anti-insulin tests. The effectiveness of the two preparations in promoting the deposition of liver glycogen in adrenalectomized mice is illustrated in table 2.

DISCUSSION. From the data presented in tables 1 and 2 it is evident that the adrenocorticotropic factor of the anterior pituitary did not exert any anti-insulin effect and also failed to promote the formation of liver glycogen in adrenalectomized mice. In normal mice a positive response has been observed as previously reported (6). Corticosterone exerted a pronounced anti-insulin response and also markedly increased the deposition of liver glycogen under identical experimental conditions. These findings are in agreement with our assumption that the anti-insulin effect of the anterior pituitary is mediated through the adrenal cortex and inhibits insulin hypoglycemia by promoting the formation of liver glycogen. They do not exclude, however, the possibility that other pituitary principles may modify insulin resistance and other phases of carbohydrate metabolism.

SUMMARY

It has been found that the adrenocorticotropic factor of the anterior pituitary failed to produce an anti-insulin effect and to promote the deposition of liver glycogen in adrenalectomized mice. On the other hand, administration of corticosterone was found to protect adrenalectomized mice against insulin hypoglycemia and to increase the amount of liver glycogen under the same experimental conditions. These results confirm our assumption that the anti-insulin effect of the anterior pituitary is produced by the adrenocorticotropic factor and is mediated through the adrenal cortex.

We wish to express our appreciation to Dr. E. C. Kendall of the Mayo Clinic for kindly supplying us with crystalline corticosterone acetate. We are also indebted to Dr. E. Schwenk of the Schering Corporation for supplying us with synthetic desoxycorticosterone acetate.

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CORTILACTIN, THE LACTATION FACTOR OF THE ADRENAL[1]

HERBERT J. SPOOR, FRANK A. HARTMAN AND KATHARINE A. BROWNELL

From the Department of Physiology, The Ohio State University, Columbus

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Adequate lactation in the adrenalectomized animal can be maintained if certain types of adrenal extract are used (1). In 1933 Brownell, Lockwood and Hartman (2) showed that such an extract could be divided into two fractions, one which maintained an adrenalectomized mother rat in good condition but would not support lactation, while the other supported lactation but had little influence on the maintenance of an adrenalectomized animal. The active factor in the latter fraction was named cortilactin.

The effectiveness of cortilactin does not appear to be limited to the rat. Thorn and Hartman (3) obtained positive effects with it in the human being.

We have been able to separate cortilactin from whole adrenal tissue by iso-electric precipitation. We have also shown that cortilactin bears no relation to gluconeogenesis.

METHODS. *Preparation of cortilactin.* We started either with the lipid precipitate obtained as a by-product in the making of ordinary adrenal extract (4) or with adrenal tissue. In the first instance the glands were extracted with 95 per cent ethyl alcohol. The alcoholic extract was concentrated to $\frac{1}{15}$ volume. The resulting concentrate was extracted with ethyl ether, the aqueous residue being discarded. The ether solution was concentrated to small volume and the residue taken up in 70 per cent alcohol. On chilling this solution to $-12°$C. a lipid precipitate was thrown down. This is the lipid precipitate mentioned above. It is dissolved at room temperature in alkaline (pH 10) 70 per cent alcohol.

When cortilactin was prepared directly from the glands, they were extracted first with alkaline (pH 10) 70 per cent ethyl alcohol. This method is preferable because separation of cortilactin from the lipid precipitate may be difficult on account of emulsification.

From either alkaline 70 per cent alcoholic solution, cortilactin was separated according to the method of Riddle, Bates and Dykshorn (5) by iso-electric precipitation in 86 per cent alcohol at a pH 5.8. The precipitate

[1] Aided by grants from The National Research Council Committee on Research in Endocrinology and the Comly Fund of The Ohio State University.

was dissolved in water or saline for administration. Potency was increased by reprecipitation.

Methods for the preparation of adrenal extract, cortin and sodium factor are described elsewhere (6).

The prolactin was dissolved in saline. The desoxycorticosterone was dissolved in 5 per cent propylene glycol saline solution.

Lactation assay. Pigeon. The pigeon crop gland response is most satisfactory for the assay of cortilactin because of the limited amount of material needed and the relatively short time required. Certain features of the assay methods of Riddle and Braucher (7) and of Lyons (8) have been combined for this test. The subjects were one month old squabs. The injections were made twice daily into the pectoral muscle for four days. On the fifth day the crop was removed, freed from adhering fat, washed and dried at 105°C. for seven hours. An increase in weight over normal control values was a measure of the effect.

Rat. Adrenal preparations proven potent by the pigeon assay were tested for their ability to support lactation in the adrenalectomized rat. These animals were maintained on the special diet which Daggs (9) used in his lactation studies. Young pregnant rats were adrenalectomized one to five days before term, after which they were maintained in good condition by adrenal extract. Only those animals which produced a normal litter were used. At birth the number of pups was reduced to six. The first seventeen days *post partum* give a direct measure of milk secretion (9), therefore the observations were not extended beyond this period.

Rat test for gluconeogenesis. The gluconeogenetic power of cortilactin was tested in the rat (10). Twelve young males of about 150 grams in weight were fasted for 24 hours. Every hour for the last seven hours of the fast four of these animals were injected subcutaneously with 1.3 mgm. of cortilactin (second iso-electric precipitate) in 1 cc. of physiological saline. At the eighth hour of the fast the extract was given intraperitoneally. A second four were injected with equal quantities of physiological saline. At each injection, the third four were given 0.06 mgm. of corticosterone in 1 cc. of 5 per cent propylene glycol in physiological saline. One hour after the last injection the animals were anesthetized with nembutal and the livers rapidly excised and dropped into 30 per cent potassium hydroxide. Liver glycogen was determined by the method of Good, Kramer and Somogyi (11).

RESULTS. *Pigeon crop response. Controls.* The birds varied from 250 to 470 grams in weight. The dry crop did not necessarily parallel the body weight. Groups of normal birds were run with each test.

The dry crop weights of 36 normal birds ranged from 0.39 to 0.87 gram with an average of 0.61 gram.

Prolactin. Purified prolactin in doses of $\frac{1}{2}$ to 1 mgm. (1 mgm. contained

6 Riddle units) daily gave definite enlargement of the crop. Comparison of the effects of various preparations is shown in figure 1.

Adrenal preparations. A few birds were tested with unfractionated or whole adrenal extract, extract containing cortin, extract containing sodium factor and desoxycorticosterone. All were negative. Figure 1 shows the doses employed.

The first precipitate prepared from the 70 per cent alcoholic direct extraction of the adrenal tissue (called "glands" in fig.) was somewhat better than the first precipitate (called "lipid" in fig.) prepared from the lipid by-product of ordinary extract preparation. The potency was doubled by iso-electric reprecipitation (called "second" in fig.).

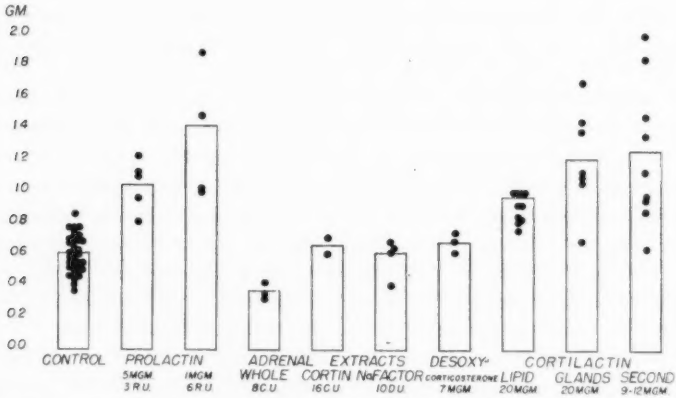

Fig. 1. Pigeon crop gland (dry weight) response to various preparations. The 20 mgm. dose made from glands was from the first iso-electric precipitation. The 9–12 mgm. dose was from the second iso-electric precipitation.

According to the pigeon test the best cortilactin preparation possessed about one-tenth of the potency of purified prolactin.

Rat lactation response. Controls. In order to ascertain the effect of the special diet on the growth of young during lactation, twenty-six normal female rats were placed on the test diet after they had become pregnant. On the day of parturition the number in each litter was reduced as indicated. The weights of the mother and litter were determined daily for 17 days. The average change in the weight of the mother was +8 per cent, ranging from −2 to +23 per cent. All young survived. The average birth weight of the individual pup was 5.3 grams ranging from 4.1 to 6.3 grams. At 17 days the average weight of the individual pup was 29.2 grams ranging from 22.5 to 34.6 grams.

Effect of adrenal preparations. Seven pregnant rats were adrenalec-

tomized just before parturition. They were injected twice daily with enough unfractionated extract (containing 8 c.u. of cortin) to maintain the mother in good condition. Four litters died, while deaths in the other litters reduced the total survival to 31 per cent. The average weight of the surviving pups on the seventeenth day was 17.1 grams.

Four adrenalectomized pregnant rats were treated as above, except that they were given double the first dose of unfractionated extract. The survival of the pups was 76 per cent with an average weight per individual of 20.9 grams at 17 days.

Six adrenalectomized pregnant rats were given treble the first dose of unfractionated extract. In this case the survival of the pups was 87 per cent with an average individual pup weight of 21.2 grams at 17 days.

Adrenal extract is particularly effective in the rat when administered orally (12). Therefore, the following experiment was tried. Three adrenalectomized pregnant rats were injected daily with 8 c.u. of unfractionated extract and in addition were given about 3 c.u. daily of unfractionated extract in their drinking water. Although the survival of the pups was 100 per cent, the average weight of the individual pup at 17 days was 17.3 grams which is far short of normal (fig. 2).

Addition of cortilactin fractions. Nine pregnant females were adrenalectomized and treated with a maintenance dose of unfractionated adrenal extract (8 c.u. of cortin daily). In addition they were injected daily with 10 mgm. of the first iso-electric precipitate (curve "cortilactin I" in fig. 2). The survival of the pups was 88 per cent as compared to 28 per cent in those pups whose mothers received no cortilactin. The average weight of the individual pup at 17 days, however, was only 17.3 grams.

Five adrenalectomized pregnant rats were injected with unfractionated extract containing 8 c.u. of cortin, given 3 c.u. of cortin orally and injected with 1 mgm. of the second iso-electric precipitate daily (curve "cortilactin II" in fig. 2). The survival of pups was 100 per cent while the average weight of the individual pup at 17 days was 28.26 grams ranging from 26.0 to 33.0 grams. This indicates complete replacement therapy.

The average growth curve of surviving pups is shown in figure 2. However as each animal died, one less was included in the average so that a curve from litters with a small percentage survival does not give a true picture of lactation effects.

In those litters where the survival was not 100 per cent, the time and amount of reduction follow: Treatment with unfractionated extract (8 c.u.)—1st day, 81 per cent; 2nd day, 79 per cent; 3rd day, 71 per cent; 5th day, 69 per cent; 8th day, 67 per cent; 10th day, 57 per cent; 11th day 53 per cent; 12th day, 43 per cent; 13th day, 41 per cent; 14th day, 33 per cent; 15th day, 31 per cent. Treatment with unfractionated extract (16 c.u.)—1st day, 96 per cent; 2nd day, 79 per cent; 6th day, 75 per cent.

Treatment with unfractionated extract (24 c.u.)—1st day, 97 per cent; 3rd day, 94 per cent; 4th day, 86 per cent. Treatment with unfractionated extract (8 c.u.) and cortilactin (cortilactin I)—1st day, 93 per cent; 2nd day, 91 per cent; 9th day, 88 per cent.

Increase in the amount of extract over that required to maintain the mother added to the number of pups surviving, but the weight of the individual pup remained low. Oral therapy supplement further increased

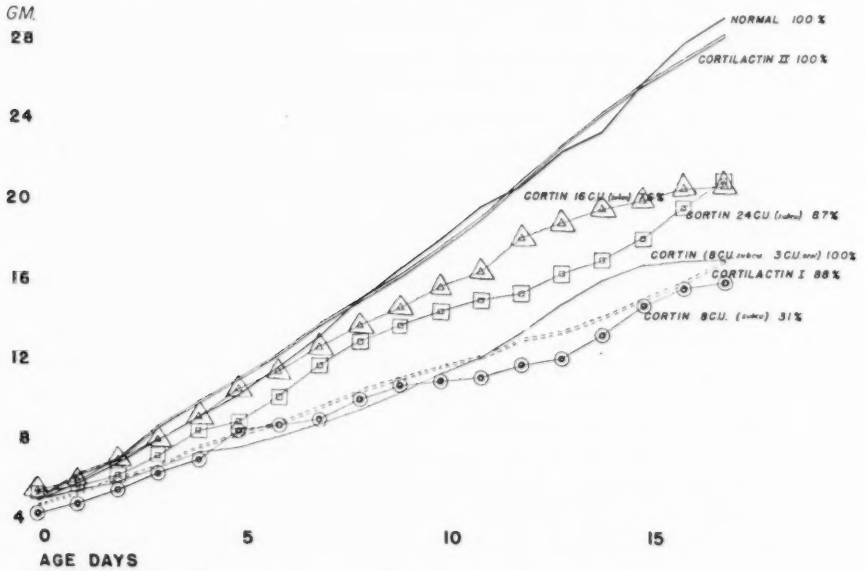

Fig. 2. Growth curves of young rats based on average age of individual pups. All injections subcutaneous except where indicated. The cortin assay is indicated as cat units in the chart. Rats treated with "cortilactin I" were also given daily subcutaneous injections of unfractionated extract containing 8 c.u. of cortin. Rats treated with "cortilactin II" were given subcutaneously, unfractionated extract containing 8 c.u. of cortin and orally, unfractionated extract containing 3 c.u. of cortin.

survival to 100 per cent with no change in pup weight. The addition of the second precipitate (cortilactin II) raised the pup weight to normal.

Effect of cortilactin on gluconeogenesis. Four cortilactin injected rats showed an average of 0.04 per cent liver glycogen, the values ranging from 0.01 to 0.07 per cent. The liver glycogen of the four saline controls averaged 0.16 per cent with a range of 0.06 to 0.30 per cent, while that of the four corticosterone controls averaged 4.18 per cent ranging from 3.55 to 5.96 per cent.

Discussion. Carr's (13) failure to support lactation in adrenalectomized rats by means of adrenal extract prepared by Swingle and Pfiffner's method may have been due to inadequate dosage. Later work of Swingle and Pfiffner (1) showed that their preparation maintained adequate lactation in the dog. Therefore dosage or species difference must have been the explanation.

The "unfractionated adrenal extract" which we used to maintain the adrenalectomized mother rats contained both cortin and sodium factor but little or no cortilactin.

Our cortilactin preparations were made from whole adrenal glands. Therefore whether cortilactin was present in either cortex or medulla or both was not indicated. However the preparations of Brownell, Lockwood and Hartman (2) were made entirely from dissected cortex. Cortex must, therefore, be a source of cortilactin. Our normal animals showed much better survival than did those of Brownell et al. (2). This was due to a better diet and to reduction of the number in the litter to six.

Gaunt and Tobin (14) found that a dosage of extract twice that necessary to maintain adrenalectomized mother rats was adequate for survival and growth of the pups. However they reduced their litters to four instead of six animals.

The iso-electric precipitation method of Riddle, Bates and Dykshorn (5) has yielded lactogenic substances from liver, blood and urine according to Ehrhardt and Voller (15) and Cunningham et al. (16).

The observation that cortilactin will stimulate crop gland formation and that it can be prepared by the same method used for prolactin, raises the question as to whether they are identical. This is answered by Gaunt and Tobin (14) who found that prolactin had no effect on lactation in the adrenalectomized rat.

We have no suggestion to offer as to the mode of action of cortilactin. It apparently has nothing to do with gluconeogenesis.

We wish to thank Dr. Oliver Kamm of Parke, Davis and Company for adrenal glands, Mr. H. W. Rhodehamel of Eli Lilly and Company for prolactin and Dr. R. D. Shaner of Roche-Organon Incorporated for desoxycorticosterone.

SUMMARY

A lactation factor has been prepared from the adrenal by iso-electric precipitation. The pigeon crop gland response was used for its assay. The best cortilactin preparation possessed about one-tenth of the potency of purified prolactin when tested by the crop gland method. The daily injection of 1 mgm. of the cortilactin preparation into an adrenalectomized rat maintained on cortin enabled her to lactate normally. The pup sur-

vival was 100 per cent and their average weight was normal. Cortilactin plays no rôle in gluconeogenesis.

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CHANGES OCCURRING IN THE BLOOD AND TISSUE OF CHICKENS DURING COCCIDIOSIS AND ARTIFICIAL HEMORRHAGE[1]

S. H. WAXLER

From the Departments of Zoology and Veterinary Science, University of Wisconsin

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Studies on the blood of chickens infected with cecal coccidiosis show a marked increase in the blood sugar. The initial rise is manifest at the beginning of the fifth day at which time the internal hemorrhage due to the disease also begins. As the bleeding becomes more profuse, the blood sugar value continues to rise until the seventh day of infection when the disease has run its course. The loss of blood has been indicated by Pratt (1940) to be responsible for the blood sugar increase during the hemorrhagic phase of the disease. The author (1941), in attempting to replace the deficient constituents of the circulating fluid with concentrated physiological saline, obtained a lower blood sugar level than ordinarily present during coccidiosis. This presented the possibility that the increased blood sugar value during an infection might be in some way related with the chlorides of the blood.

An experiment was undertaken to study the relationship of the sugar and chloride and the changes brought about by coccidiosis: 1, the blood chlorides and sugars were determined before and during the course of an infection; 2, sugar and chloride determinations were made following artificial hemorrhage and these results were compared with those of coccidial bleeding; 3, gum acacia solution was injected in order to replace the blood volume which was lost during artificial bleeding and the resulting chloride values were observed; 4, tissue chloride determinations were obtained from normal and infected animals.

MATERIALS AND METHODS. The experimental animals were Single Comb White Leghorn chickens furnished by the Department of Poultry Husbandry of the University of Wisconsin. The chicks, obtained when one day old, were kept free of infection in sterilized cages and given the feed and care described by Herrick, Ott and Holmes (1936). The chickens were

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infected orally, by means of a pipette, with about 1 cc. of a pure culture of *Eimeria tenella* containing approximately 200,000 viable coccidia oöcysts. The birds were off-feed 19 hours preceding all determinations in order to obtain a basal level from which to measure the changes due to the disease. This procedure was necessary to remove the fluctuations brought about by *ad libitum* feeding.

The blood and tissue chloride determinations were carried out according to the procedure of Van Slyke (1923). The chloride concentrations have been expressed in the terms of NaCl. The blood sugar values were determined colorimetrically by the Folin-Wu method (1920) with an Evelyn photoelectric colorimeter. The blood samples were obtained in all cases by cardiac puncture. The animals were not anesthetized and no anticoagulants were used since the blood was tested as soon as it was drawn. The tissue samples were taken in duplicate and triplicate from the right and left pectoral muscles.

The gum acacia which was used for transfusions was a sodium chloride-free preparation made by Lilly. The gum acacia was made up to a 6 per cent solution by diluting with distilled water. The chloride-free compound was used so that the effect of the transfusion could be judged by the changes in the chloride value. Transfusions were made directly into the heart with a syringe.

Experiments. 1. *Blood chloride and sugar changes during cecal coccidiosis.* The animals used in this experiment were divided into two groups; one was infected, the other was not and served as a control. The chloride values of both were obtained before oöcysts were administered and then again on the 4th, 5th, 6th and 7th days following infection. According to the data of table 1, the chickens before infections collectively averaged 472 mgm. of sodium chloride per 100 cc. of blood. On the fourth day the infected group averaged 465 mgm., and on the 5th day was also slightly lower than the preinfection value. A rise was manifested on the 6th day and continued upward to 550 mgm. on the 7th day of the infection. The values of the uninfected controls remained fairly close to the preinfection level of 472 mgm. The chlorides of these animals averaged 465, 467, 454 and 478 mgm. from the 4th through the 7th day respectively. At this time, coccidiosis had effected an average rise of 86 mgm. in two days.

To ascertain whether any relationship existed between this rise in the chlorides and that of the blood sugars during coccidiosis, the two were graphed. The chlorides of the above experiment were compared with the blood sugars of a typical case of coccidiosis. The blood sugar values are from the author's (1941) previous experiment. Before infection the average sugar content of the blood was 175 mgm. per 100 cc. of blood. The values from the 4th through the 7th day show a definite rise as follows: 185, 236, 259 and 251 mgm. per 100 cc., an average increase of 76 mgm.

When the blood chlorides were graphed against the blood sugars, it was apparent that the increase in the chlorides occurred at least a day following that of the sugar. The sugar of the blood showed a definite ascent by the 5th day, reached a peak on the 6th day, and then began to taper off. The chlorides remained fairly close to the control value until the 6th day, when a significant increase was noted, and then continued their rise through the 7th day. It appeared that the mechanism operative in producing the rise allowed for a lag of a day for the chlorides in comparison with that of the sugars.

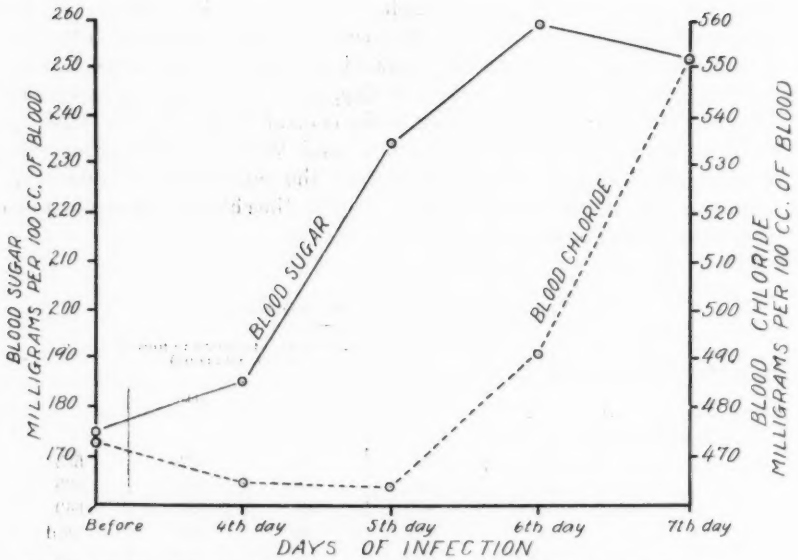
TABLE 1

Effect of coccidia on blood chlorides

CHICKEN NUMBER	BEFORE INFECTION	DAYS AFTER INFECTION (RESULTS IN MGM. OF NaCl PER 100 CC. OF BLOOD)			
		4th	5th	6th	7th
Infected					
1	485	437	454	500	558
2	434	460	449	446	536
3	482	482	467	460	549
4	487	470	464	536	Dead
5	477	474	487	512	556
Uninfected					
6	457	455	447	454	477
7	460	460	469	444	502
8	477	483	482	422	437
9	482	459	469	464	483
10	477	469	467	485	492
Résumé					
Infected	473	465	464	491	550
Uninfected	471	465	467	454	478

2. *Blood chloride and sugar changes following artificial hemorrhage.* The second phase of the experiment concerned the chloride changes that occurred following artificial hemorrhage. By such a procedure Pratt (1940) was able to obtain a rise in the blood sugar similar to that produced by an infection of coccidia. This portion of the work was undertaken to determine whether this type of bleeding would promote a rise in the chlorides in the same way as an infection.

The hemorrhage was produced by withdrawing the blood by direct cardiac puncture. Thirteen chickens of various ages and weights were tested by this procedure. The amount of blood which was taken varied according to the size of the bird and the quantity which could be removed by one puncture without distressing the animal. The average volume that



BLOOD SUGAR AND CHLORIDE CHANGES DURING CECAL COCCIDIOSIS

TABLE 2

Effect of artificial bleeding on blood sugar and chloride

NUMBER	WEIGHT	AMOUNT BLED	BLOOD CHLORIDE		BLOOD SUGAR	
			Before	After	Before	After
	gm.	*cc.*	*mgm. per 100 cc. of blood*			
1	1330	39	478	519	180	245
2	1420	37	462	486	162	239
3	1580	38	478	503	181	259
4	1420	42	453	528	173	259
5	1490	32	511	543	195	261
4*	873	23	442	528	199	244
5*	790	25	497	543	198	247
6	1290	30	503	536	168	228
7	740	25	486	557	196	240
8	838	25	478	561	192	246
9	840	15	483	519	207	225
10	884	24	445	536	186	268
11	886	23	453	536	182	260
Average			474	530	186	248

* Tested two months previous.

was withdrawn was 28 cc., the minimum 15 cc., and the maximum was 42 cc. The chloride content was determined at the time of bleeding and three hours later. The difference between the two was considered as due to the removal of blood. Blood sugar determinations were also made at the same time.

The data from table 2 show that with bleeding the sodium chloride content increased from an average of 474 to 530 mgm. per 100 cc. of blood, an average rise of 56 mgm. This change compared favorably with the 72 mgm. increase obtained with an infection of coccidia (see table 1). At the same time the blood sugar level was in line with that of an infection, as graphed above. The results tended to indicate that the rises were due to the loss of blood in both cases.

An examination of two of the birds (nos. 4 and 5) indicated the constancy of the results which were obtained. These two chickens had been tested two months previously. In regard to no. 4, the chlorides increased at the initial bleeding from 453 to 528 mgm. Two months later, the determinations were 442 mgm. before and 528 mgm. after bleeding. Results of fairly similar uniformity were also obtained for the blood sugars.

3. *Blood chloride level following artificial hemorrhage and gum acacia injection.* It was apparent that the rise in blood chloride and blood sugar brought about by an infection of coccidiosis could be simulated by artificial hemorrhage. To further emphasize that this rise in the chlorides with artificial bleeding and with infection was due to loss of blood, an experiment was set up to replace the deficient volume in artificially bled animals. In a previous experiment, the author had attempted such a procedure by feeding concentrated physiological saline solution to infected animals. This was accomplished when the birds drank excessively of water and showed a lowered blood sugar level. It was thought that this was due to excess water intake and blood volume dilution. In this experiment, the deficient volume was replaced directly by 6 per cent sodium chloride-free gum acacia.

These animals were bled by a single direct cardiac puncture and the blood withdrawn within 10 seconds or less. Immediately thereafter, in some cases without removal of the needle, the gum acacia was injected into the heart. About two-thirds of the volume of blood that had been withdrawn was replaced by the injection of the 6 per cent gum acacia solution. Six chickens were so treated and the results recorded in table 3.

The data indicated that when the gum acacia injections were given after excessive bleeding, no rise in the blood chlorides occurred. No changes were noted after a three hour period, or even after 24 hours had elapsed. The average chloride content of the blood was 481 mgm. before and after the bleeding with gum acacia treatment. The individual differences at the two bleedings were also very close.

4. *Tissue chlorides of infected and normal chickens.* It was apparent

from the previous experiments that there was a rise in the blood chlorides with artificial bleeding as well as with coccidia hemorrhage. There is no single salt depot in the body upon which the chicken may draw to raise this chloride content. The only probable source of chloride which is available is that of the tissue. With this in mind, an experiment was conducted to determine the tissue chlorides of normal and infected animals. The methods for tissue chlorides are known to be unreliable and so deter-

TABLE 3

Six per cent sodium chloride-free gum acacia after mechanical bleeding

NUMBER	WEIGHT	AMOUNT BLED	GUM ACACIA	BLOOD CHLORIDES (RESULTS IN MGM. PER 100 CC. OF BLOOD)		
				Before	After	24 hours later
	gm.	*cc.*	*cc.*			
1	1030	28	18	479	477	477
2	1005	25	16	464	472	464
3	1195	30	20	479	477	472
4	1100	30	20	483	470	470
5	920	18	12	496	495	
6	800	23	16	487	495	
Average				481	481	472

TABLE 4

Effect of coccidiosis on tissue chloride

DAY OF INFECTION	DETERMINATIONS	DAILY AVERAGE	AVERAGE
		mgm. per 100 grams tissue	*mgm.*
Infected			
5	28	46.24	
6	24	47.18	
7	26	46.51	46.62
Uninfected—control			
5	26	49.33	
6	28	51.53	
7	21	51.03	50.62

minations were made in duplicate and triplicate for each animal. The uninfected controls averaged 50.62 mgm. of sodium chloride per 100 grams of tissue. A down-trend was evident in the infected birds on the 5th, 6th and 7th day after infection. The average values for these three days were as follows: 46.24, 47.18 and 46.51 mgm.

Discussion. One of the most notable changes in the blood of chickens following coccidia hemorrhage is the rise in the blood chlorides. An in-

crease of almost the same magnitude is apparent when the chickens are artificially bled. There is at present no obvious evidence of a salt deposit upon which the body can draw to account for the above increased blood chlorides. The drop in the chloride content of the tissue during an infection indicates that the increase in the chlorides of the blood may be derived from the tissue. Starling (1909) stated that after artificial hemorrhage there may be a passage of water and salts from the extra-vascular fluids into the blood vessels.

This influx of salts and water into the blood maintains the circulatory volume. Does this increase in chlorides also help to maintain the osmotic pressure of the blood, which according to preliminary determinations remains relatively constant following both types of hemorrhage? The results of injecting 6 per cent sodium chloride-free gum acacia solution also seems to point in this direction. This solution not only replaces the lost volume but also maintains the osmotic pressure by taking the place of the plasma proteins which were lost by bleeding. Bayliss (1920) indicated this by showing the expediency of injecting gum-saline rather than saline after hemorrhage in an attempt to maintain blood volume and blood pressure. In this experiment there was no rise in the blood chlorides after hemorrhage and gum acacia injection indicating, at least, that when the osmotic pressure of the blood was maintained by some other medium, the chlorides did not rise.

The increase in the chlorides with artificial hemorrhage and with coccidiosis parallels the work of Pratt (1940) with blood sugar. He showed that extensive cardiac hemorrhage artificially produced in normal chickens caused a rise in the blood sugar of the same degree as that caused by coccidiosis. He attributed this hyperglycemia to the muscle and perhaps liver glycogen. To account for the blood chloride increase of this experiment, a decrease in the tissue chloride has been indicated. Although the drop in the tissue chloride was only 4 mgm. per 100 grams of tissue, the amount which was lost by the animal tissues as a whole was quite large and could account for at least a good share of the chloride rise of the blood.

CONCLUSIONS

1. There is an increase of the blood chloride on the 6th and 7th day of an infection of cecal coccidiosis in chickens.

2. The rise in the blood sugar, due to coccidiosis, is apparent on the 5th day, a day prior to the rise of the chlorides.

3. Artificial hemorrhage produces an increase in the blood chloride and sugar approximate to that brought about by bleeding from coccidiosis.

4. The chloride content of the blood is maintained at the normal level after severe artificial hemorrhage by the injection of 6 per cent sodium chloride-free gum acacia solution.

5. The chloride content of the muscle shows a downward trend during coccidiosis and may account in part for the rise in the blood chloride.

Acknowledgments are made to Drs. C. A. Herrick and W. H. McShan for their many helpful suggestions.

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THE EFFECT OF INORGANIC IONS ON GASTRIC SECRETION IN VITRO

J. S. GRAY AND J. L. ADKISON

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago

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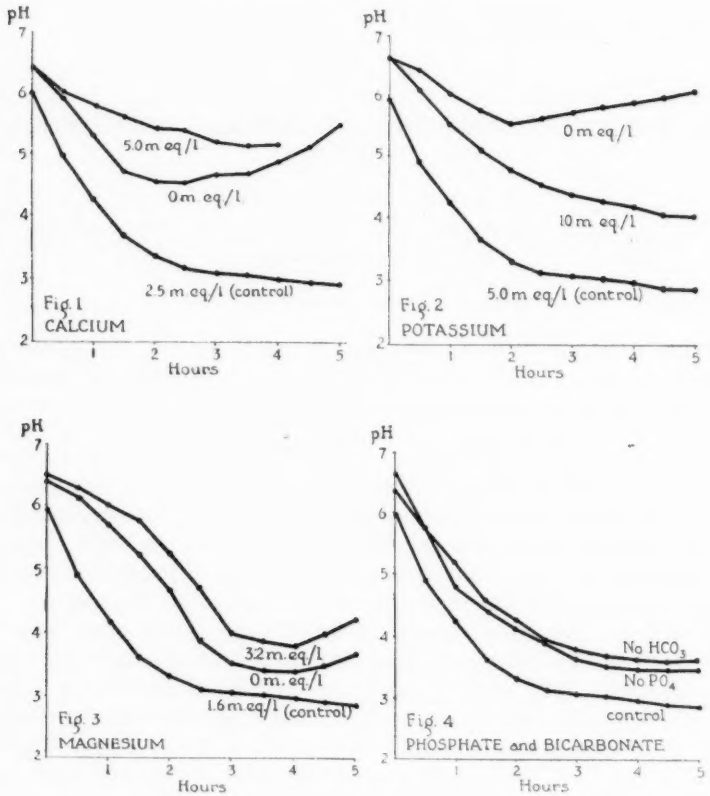
It has been reported that the isolated gastric mucosa of the frog will form acid when properly mounted in a bath consisting of two chambers separated by the gastric mucosa (1, 2). We have recently demonstrated that this *in vitro* production of acid represents a true secretory process on the part of the gastric glands (3). The present investigation is concerned with the response of the isolated glands to changes in their ionic environment. This was undertaken in order *a*, to determine whether glandular tissue resembles smooth muscular tissue in its requirements for optimum concentration of various ions, and *b*, to determine, if possible, which ions might be involved in the process of acid formation in the stomach.

METHODS. The gastric mucosa of fasted frogs was dissected from the muscularis and mounted in the dual-chambered bath exactly as previously described (3). This bath consists of one chamber filled with an isotonic salt solution which receives the secretion, and a second chamber containing a nutrient solution consisting of 71.75 mM./l. of NaCl, 20.0 of Na_2CO_3, 5.0 of KCl, 1.25 of $CaCl_2$, 1.20 of sodium phosphate buffer (pH 7.4), 0.80 of $MgCl_2$. Glucose was added in a concentration of 50 mgm. per cent. This solution was aerated with 5 per cent CO_2 and 95 per cent O_2; the secretory solution was aerated with air, mainly for purposes of stirring.

In order to determine the effect of varying the ionic composition of the nutrient solution, the various ions were successively doubled in concentration and omitted entirely. The altered ion was replaced by or substituted for an equivalent amount of NaCl in order to maintain isotonicity. When the bicarbonate ion was omitted, the nutrient solution was aerated with pure O_2, and the air bubbled through the secretory solution was freed of CO_2.

The changes in the pH of the secretory solution were followed over a period of five hours, using a glass electrode as previously described. The resulting curves were compared with a series of control curves obtained without alteration of the nutrient solution.

RESULTS. The results of the various experiments are presented in the



Figs. 1–4. The effect of alterations of ion concentration on the formation of acid by the isolated gastric mucosa of the frog.

TABLE 1

Average, maximum, and minimum low pH values

	CONTROL	Ca		K		Mg		PO_4 OMITTED	HCO_3 OMITTED
		Omitted	Doubled	Omitted	Doubled	Omitted	Doubled		
Number of trials	11	8	8	8	6	4	4	8	12
Average	2.89	4.51	4.90	5.49	3.83	3.38	3.68	3.43	3.48
Maximum	3.22	6.42	5.72	6.50	5.01	3.52	4.07	4.60	4.58
Minimum	2.54	3.39	3.71	3.51	3.07	3.21	3.21	2.87	1.49

figures and the table. In the former, the average pH curves over the five hour period are shown for each type of experiment; in the latter, the average, together with the maximal and minimal values for the lowest pH attained in the individual trials for each type of experiment are shown.

In the eleven control experiments the curve reached an average low level of pH 2.9 at the end of five hours. In the individual trials the low point ranged from 3.22 to 2.54. The degree of uniformity that is encountered in this type of experiment is indicated by the close similarity between these eleven entirely new and the eight previously published control curves (3).

When the calcium ion was entirely omitted from the nutrient solution, the pH fell more slowly, did not fall as far, and began to rise again before the conclusion of the five hour period. A similar, but more marked, interference with acid formation occurred when the calcium concentration was doubled.

In the case of the K ion, its omission seriously interfered with secretion, whereas doubling its concentration produced less extensive inhibition. Alteration in either direction of the Mg ion concentration produced mild inhibitory effects which consisted mainly of delaying the onset of secretion.

The ions whose absence appeared to have the least injurious action were found to be PO_4 and HCO_3, the two ions which are generally considered to be involved in the formation of HCl by the gastric glands. The lowest pH which has so far been recorded, namely, 1.49, occurred in the absence of the HCO_3 ion.

Discussion. Variations in the ionic composition of the artificial bath affect the *in vitro* secretion of acid by the gastric glands of the frog. In this respect the mucosa is most sensitive to variations in Ca ion, less so to K ion, still less to Mg ion, and scarcely at all to PO_4 and HCO_3 ions. With the single exception of the HCO_3 ion, this series is identical with that demonstrated by Van Dyke and Hastings (4) to hold for uterine smooth muscle *in vitro*. Delrue (2) who employed the isolated gastric mucosa of the frog reported the following series, Ca, HCO_3, K and PO_4. His experiments, however, were performed in the late fall, so that the pH of the secretory solution rarely fell below 5.0. The primary importance of the calcium ion for gastric secretion is also indicated by reports that gastric secretion *in vivo* is inhibited by alteration in either direction of its concentration in the blood (5, 6, 7, 8, 9, 10).

At first glance, the failure of the absence of PO_4 ions to interfere with secretion *in vitro* appears to be contrary to Maly's hypothesis concerning the formation of HCl. However, since the PO_4 ion is not used up in Maly's reactions, a normal initial supply may serve the requirements of

the cell for this ion. Accordingly, the present observations neither support nor deny Maly's hypothesis.

The finding that the omission of the HCO_3 ion does not interfere with the *in vitro* formation of acid is in contradiction to the generally accepted view. There have been a number of reports to the effect that a reduction in blood bicarbonate inhibits gastric secretion (11, 12, 13, 14). In most cases this reduction has been accomplished by hyperventilation, which can reduce the ionic calcium of the blood to the point of tetany. Furthermore, the inhibition of secretion is difficult to demonstrate against a stimulus as potent as histamine (14). The use of the isolated mucosa makes it possible to distinguish between direct and indirect effects of various procedures on the gastric glands, and in this case it would appear that the effects of reduction of the blood bicarbonate are expressed indirectly, since they do not occur *in vitro*.

It would seem unlikely that a cell which requires considerable energy to form its secretory product should be dependent upon an *external* source of CO_2 or bicarbonate, for its own metabolism must supply large amounts of this substance. It has recently been demonstrated that the mammalian parietal cell contains the enzyme, carbonic anhydrase, in greater concentration than the red cell (15, 16). It is probably the function of the enzyme to convert carbon dioxide gas, formed within the cell in large quantities, into ionizable carbonic acid, of which the H ion becomes available for the formation of acid, and the HCO_3 ion for release to the blood stream in exchange for Cl ions. Accordingly, there seems to be no *a priori* reason for believing the acid forming cells to be dependent upon an outside source of CO_2.

CONCLUSIONS

1. The gastric glands of the frog *in vitro* resemble smooth muscle in their sensitivity to alteration in their ionic environment. The descending order of sensitivity to the various ions is as follows: Ca, K, Mg, PO_4 and HCO_3.

2. An *external* supply of PO_4 and HCO_3 ions is not necessary for the formation of acid by the gastric glands of the frog *in vitro*.

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GASTROINTESTINAL TRACT MOTILITY IN THE ABSENCE OF BILE

ROBERT F. ACKERMAN, HOWARD CURL AND LATHAN A. CRANDALL, JR.

From the Departments of Physiology and Anatomy, University of Tennessee College of Medicine, Memphis

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A knowledge of the laxative effect of bile was part of the medical lore of the ancient world. The modern contributions to this subject have recently been reviewed by Haney, Roley and Cole (1), who conclude as a result of their own studies on dogs with Thiry-Vella loops that the introduction of bile stimulates the motor activity of the small intestine and that this effect is due entirely to the presence of bile salts. They further suggest that "bile salts may play an important rôle in the normal regulation of the propulsive movements of the small intestine."

While the evidence presented by Haney et al. that bile salts applied to the mucosa of isolated loops will increase loop motility can hardly be questioned, the rôle of the normal biliary secretion in the activation of intestinal motility seemed to deserve further study. Peters (2) has shown that bile salts, in the concentration in which they occur in bile, inhibit absorption from the ileal loops and that this concentration is not normally present in the ileum. That the ileum may be in general less resistant to irritation than the upper small bowel is also indicated by the observations of Dennis (3). Haney et al. do not state the origin of the loops used by them, but if they were segments of ileum one may question the propriety of drawing inferences concerning normal digestive tract behavior from the effects of undiluted bile on such loops.

We have carried out roentgenologic studies in bile fistula dogs using meals containing large and small proportions of fat in order to determine under more physiological conditions the relationship of bile to gastrointestinal motility.

METHODS. Bile fistulae of the "internal" type were made in 8 dogs, using the technique of Kapsinow, Engle and Harvey (4) (anastomosis of gall bladder to right renal pelvis with ligation of the common duct). The animals were maintained on a diet consisting of meat scrap 8, corn meal 30, soy bean meal 50, wheat germ 10, alfalfa leaf meal 1.5, and salt 0.5; the fat content of this diet is approximately 5 per cent. They received parenteral injections of vitamins A, D, E and K since it is known that in

the absence of bile the absorption of the last three of these and of carotene is inadequate.

Two types of barium meals were used. No. 1, a mixed meal, was composed of 100 grams barium sulfate, 100 grams ground beef, and 100 ml. milk. No. 2 (fat meal) consisted of 30 grams barium sulfate, 100 ml. Wesson oil and 100 ml. water. Meal no. 1 was fed to 8 normal and 7 bile fistula dogs, and x-ray films were made 4 and 8 hours after feeding. Meal no. 2 was given by stomach tube to 5 normal and 7 bile fistula dogs, films being made at $4\frac{1}{2}$ and $7\frac{1}{2}$ hours.

TABLE 1

Proportions of meal no. 1 present in various parts of gastrointestinal tract 4 and 8 hours after feeding

NORMAL DOGS				BILE FISTULA DOGS				
Dog number	Stomach	Small intestine	Large intestine	Dog number	Time since operation	Stomach	Small intestine	Large intestine
4 hours								
1	3/6	3/6		1B	2 weeks	3/6	1/6	2/6
2	3/6	3/6		2B	6 weeks	2/6	3/6	1/6
3	4/6	2/6		3B	3 months	2/6	4/6	
4	4/6	2/6		4B	11 months	3/6	3/6	
5	3/6	3/6		5B	4 months	2/6	2/6	2/6
6	3/6	3/6		6B	4 months	2/6	3/6	1/6
7	3/6	3/6						
8 hours								
1	2/6	2/6	2/6	1B		tr	2/6	4/6
2	2/6	1/6	3/6	2B			2/6	4/6
3	1/6	2/6	3/6	3B		1/6	2/6	3/6
4	2/6	1/6	3/6	4B		1/6	2/6	3/6
5	1/6	1/6	3/6(E)	5B		1/6	2/6	3/6
6	1/6	1/6	4/6	6B		1/6	2/6	3/6
7	1/6	2/6	3/6					

The dogs used as controls had in every instance been kept in the laboratory on our stock diet for several weeks.

Results. In order to compare the progress of the barium meal in the various animals, the proportions of the meal present in stomach, small intestine, and large intestine were estimated by inspection of the x-ray films. For convenience, the larger amount of barium in meal no. 1 was expressed in sixths, and that in the smaller meal in fourths. The results are given in tables 1 and 2.

We have regarded differences between the normal and bile fistula animals as being significant only when at least 50 per cent of the bile fistula

dogs showed rates of barium movement outside of the limits of variation found in the normal series. On this basis it may be concluded that after a large meal (no. 1) the stomach of the bile fistula dog empties more rapidly and that there is earlier entry of barium into the colon (4 hr. films). There is also evidence in the 8 hour films of more rapid gastric emptying in the bile fistula dogs, but the change from the normal is not definitely significant.

The 4½ hour films taken after the administration of the fat meal (no. 2) show no indubitably significant difference between normal and operated

TABLE 2

Proportions of fat meal (meal no. 2) remaining in various parts of gastrointestinal tract 4½ and 7½ hours after feeding

NORMAL DOGS				BILE FISTULA DOGS						
				MEAL ALONE				MEAL PLUS 3 GRAMS BILE SALTS		
Dog number	Stomach	Small intestine	Large intestine	Dog number	Stomach	Small intestine	Large intestine	Stomach	Small intestine	Large intestine
4½ hours										
8	1/4	3/4		1B		1/4	3/4	1/4	1/4	2/4
9	2/4	1/4	1/4	2B	4/4	tr		1/4	2/4	1/4
10	tr	4/4		3B	1/4	2/4	1/4	1/4	3/4	
11	2/4	2/4		4B	3/4	1/4		2/4	2/4	
12	2/4	2/4		5B	2/4	2/4		1/4	1/4	1/4(E)
				6B	2/4	2/4		1/4	3/4	
7½ hours										
8	tr	2/4	2/4	1B		tr	4/4		tr	4/4
9		1/4	3/4	2B	2/4	1/4	1/4	1/4	tr	3/4
10		2/4	2/4	3B		1/4	3/4		2/4	2/4
11	tr	2/4	1/4(E)	4B	3/4	1/4		1/4	2/4	1/4
12	1/4	3/4	tr	5B	1/4	2/4	1/4	1/4	2/4	1/4
				6B	2/4	2/4	tr	tr	2/4	2/4

animals although the barium tends to remain longer in the stomach and to pass more rapidly through the small intestine in the latter. But in the 7½ hour films it is apparent that gastric emptying is definitely slower in the bile fistula dogs. The addition of 3 grams of bile salts to the fat meal increased gastric emptying in the bile fistula dogs so that it fell within normal limits.

It should be noted that the small intestine of the bile fistula dogs contained, on the average, less barium than did that of the normal animals in every series of films except those taken 8 hours after meal no. 1. This

constant difference may indicate a greater irritability of the small bowel in the operated animals.

It has been impossible to correlate the observed changes in motility with post-operative time or with the general condition of the animals. One bile fistula dog was omitted from the series because roentgenologic observations indicated the presence of adhesions about the duodenum which accounted for a greatly increased gastric emptying time in this animal.

Discussion. The increased rate of gastric emptying of the bile fistula dogs after a mixed meal was unexpected and is not easily explained. Fauley and Ivy (3) observed a similar decreased stomach emptying time after ligation of the pancreatic ducts or extirpation of the pancreas; they suggest that the increased appetite of their animals may be responsible, since it is known that the presence of hunger augments gastric evacuation, but point out that unknown factors may be concerned. Some of our bile fistula dogs ate well but others did not, and we can find no indication of any correlation between gastric evacuation and appetite in our series.

The delayed gastric emptying after a fat meal in the bile fistula dog may be more readily accounted for on the basis of known mechanisms. It seems probable that delayed absorption of fatty acids due to the absence of bile would lead to an increased formation of enterogastrone and a greater inhibition of gastric motility. Decreased motility after the fat meal is exhibited only by the stomach and not by the small intestine. The restoration to normal rates by the administration of bile salts is to be expected on this basis.

In general our data lend no support to the concept that bile salts are an important regulator of small intestinal activity. The increased motility produced by the contact of whole bile with isolated intestinal loops (1) is probably attributable to chemical irritation from concentrations of bile salts exceeding those present in the bowel under normal conditions.

It is interesting to speculate on the possible relationship of the increased rate of gastric emptying in the bile fistula dogs to ulcer formation. Some 15 per cent of these animals are known to develop peptic ulcer (6). In view of the known relationship of mechanical factors to ulcer (7), does an increased gastric motor drive secondary to an absence of bile from the intestine contribute to ulcer development?

CONCLUSIONS

1. Bile fistula dogs given a mixed meal exhibit an increase in rate of gastric evacuation and of entrance of barium into the large intestine.

2. After a fat meal, the rate of gastric emptying in the bile fistula preparation is slower than in normal dogs, possibly because of the formation of increased amounts of enterogastrone.

3. The administration of bile salts with the fat meal brings the gastric emptying time of the bile fistula dog to within normal limits.

4. The suggestion that bile salts are an important factor in the regulation of small intestine motility is not supported by our observations.

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FURTHER EVIDENCE FOR THE FLUID CIRCUIT THEORY

The Rate of Chloride Accumulation in the Lower Ileum under Conditions Suitable for Active Chloride Absorption

H. C. PETERS

From the Department of Physiology, University of Tennessee, Memphis

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In an attempt to explain the absorption of chloride from the lower ileum which occurs against concentration gradients, Ingraham, Peters and Visscher (1) proposed the "fluid circuit" theory, which assumes that the absorption of water carries chloride out of the intestinal contents without changing its concentration from that in the lumen while a fluid free of chloride moves from the blood into the intestinal lumen. The theory has been supported by many experiments (1, 2). Most of these, however, would lose their significance if the passage of considerable amounts of chloride from blood to lumen could be demonstrated under similar conditions. The author has therefore studied the accumulation of chloride in initially chloride free solutions in the lower ileum under conditions duplicating as closely as possible those of the previous experiments.

Methods. Dogs were anesthetized with sodium amytal injected intraperitoneally, 65 mgm. per kgm. In experiments 5 and 6 this was supplemented with small amounts of ether. A loop of lower ileum about 20 inches long and about 6 inches from the cecum was isolated. A rubber cannula was inserted into one end of the loop and a glass cannula in the other. It was then rinsed out with 3 to 4 liters of isotonic NaCl at about 37° until the washings were clear. A rubber plug, having a longitudinal hole nearly but not quite through it for insertion of a needle, was then substituted for the glass cannula, and the loop was again rinsed out to remove traces of blood. After the rubber cannula had been closed with a short length of glass rod, the loop was returned to the abdominal cavity and allowed to rest for 30 minutes. The dog was kept warm with an electric lamp during the entire experiment.

The solutions used are described in the legend for figure 1. In 6 out of 8 experiments they are the same as the solution of $\frac{1}{2}$ isotonic Na_2SO_4 and $\frac{1}{2}$ isotonic NaCl used formerly (2) except that all or nearly all of the NaCl has been replaced by glucose, which was chosen because it is non-irritating and easily absorbed. At the end of the rest period 50 cc. of the

experimental solution at 37° was injected from a syringe into the loop through the rubber cannula. A hypodermic syringe and needle were used at intervals to puncture the rubber plug and withdraw 2 cc. samples. The intestinal contents were gently mixed just before sampling. During all procedures an effort was made to avoid mechanical stimulation of the intestine. Chloride concentrations were determined by the method of Van Slyke (3).

Results. Chloride concentration is plotted against time in figure 1. In no case was a concentration greater than 0.032 per cent NaCl observed. Water absorption was satisfactory in all experiments and averaged 28 cc. per hour. Active chloride absorption occurred at some time during the experiment whenever sulfate was present. In the other experiments active chloride absorption was not demonstrated.

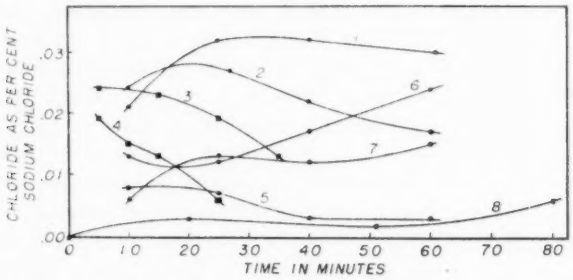

Fig. 1. Accumulation of chloride in the lower ileum. The isotonic solutions used were as follows: for experiments 1, 2, 5 and 8, ½ isotonic Na_2SO_4 and ½ isotonic glucose; for experiments 3 and 4, 44/90 isotonic Na_2SO_4, 44/90 isotonic glucose, and 1/45 isotonic NaCl; for experiments 6 and 7, isotonic glucose.

Discussion. Chloride accumulation in the lower ileum has been previously studied under different conditions. Cohnheim (4) obtained values ranging from 0.035 to 0.22 per cent NaCl. Frey (5, 6) found concentrations of from 0.03 to 0.09 per cent NaCl. In a single experiment Burns and Visscher (7) obtained low values, but chloride concentrations during the first hour were not determined.

The definitely lower rate of chloride accumulation reported here seems to be the result of the special conditions chosen. These include as the most important factors an isotonic non-irritating solution, a 30 minute rest period, minimal mechanical stimulation, and optimal anesthesia. Cobet (8) and Dennis and Visscher (9) have shown that anesthesia promotes active chloride absorption, at least in some dogs.

The present studies fail to reveal any facts which would invalidate or weaken previous evidence for the fluid circuit theory. Therefore reasonable objection to the theory on the basis of accumulation experiments

in the literature is no longer possible. However, the experiments reported here do not constitute actual proof that considerable amounts of chloride do not pass from the blood into the intestinal lumen. It is conceivable that rapid absorption might prevent accumulation in spite of such transfer.

The author (10) has recently developed a general fluid circuit theory which includes diffusion and secretion of chloride and osmosis. Although the differential equation arrived at is rather complicated, some simplification has resulted from the use of the variable, C_e, the effective concentration of chloride in the fluid passing into the intestinal lumen. According to the theory, if the slope of the concentration-time curve, dc/dt, is negative, C_e is less than the concentration in the loop at the time. Examination of figure 1 will demonstrate that wherever this principle can be applied, C_e is less than 0.033 per cent NaCl. If we consider the earliest negative slopes, we find an average upper limit for C_e of 0.018 per cent NaCl. The use of the original simple form of the fluid circuit theory in the earlier experiments therefore appears to be justified. The general fluid circuit theory should be applied when movement of chloride into the intestinal lumen or osmosis is not negligible.

SUMMARY

Under the conditions of these experiments chloride accumulates very slowly in originally chloride free solutions in the lower ileum. The highest concentration observed was 0.032 per cent NaCl.

The average upper limit of the effective chloride concentration of the fluid entering the intestinal lumen was 0.018 per cent NaCl according to the general fluid circuit theory.

The results support previous experiments on the fluid circuit theory.

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THE RELATION BETWEEN THE PHOSPHATE CHANGES IN BLOOD AND MUSCLE, FOLLOWING DEXTROSE, INSULIN AND EPINEPHRIN ADMINISTRATION[1]

SAMUEL SOSKIN, R. LEVINE AND O. HECHTER

From the Department of Metabolism and Endocrinology[2], Michael Reese Hospital, and the Department of Physiology, University of Chicago

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It has been well established that the administration of either dextrose, insulin or epinephrin to normal animals causes a decrease in the inorganic phosphate of the serum or whole blood (1, 2, 3, 4). It has also been shown that there is an increase in the hexose monophosphate content of skeletal muscle after insulin and epinephrin injections (1, 5). Since the concept was first advanced by Cori (1), it has been generally assumed that the inorganic phosphate which leaves the blood under the above circumstances enters the muscle together with blood glucose to form the muscle hexose monophosphate which appears. The fact that insulin which lowers the blood sugar and epinephrin which raises it both cause a fall in blood inorganic phosphate has been explained on the basis of experiments on adrenalectomized animals. In the latter, Cori found that insulin did not decrease the muscle hexose monophosphate while epinephrin still produced its usual effect. He concluded that when insulin acts on the blood and muscle phosphates in the normal animal, it does so by causing a reflex epinephrin secretion consequent to the hypoglycemia which the insulin induces.

Several authors have noted serious and hitherto unexplained objections to the above concepts:

1. The administration of sugar causes a fall in blood inorganic phosphate, but does not change the hexose monophosphate level in the muscle (1, 2).

2. The administration of sufficient sugar simultaneously with insulin to avoid the reflex secretion of epinephrin due to hypoglycemia results in a fall in blood inorganic phosphate without a change in the muscle hexose monophosphate (1, 2).

3. In adrenalectomized animals, in which Cori (5) found that insulin did not increase muscle hexose monophosphate, Ellsworth and Wein-

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stein (6) nevertheless reported that the serum inorganic phosphate was lowered to the same degree as in normal animals.

4. Pollack et al. (7, 8), who demonstrated that glucose and insulin caused a fall in the serum inorganic phosphate of blood perfused through the isolated limbs of dogs, failed to discover any change in the hexose monophosphate content of the perfused muscles.

5. In the perfused hind limbs of cats Lundsgaard (9) found that the active deposition of glycogen in the muscles following insulin addition, was accompanied by a fall in the inorganic phosphate of the perfusing blood. However, when glycogen deposition was accomplished by raising the dextrose level without the addition of insulin, no change in the blood inorganic phosphate occurred.

The present work accounts for the apparent inconsistencies noted above, and offers a satisfactory explanation for the phosphate changes in blood and muscle which follow the administration of dextrose, insulin and epinephrin.

METHODS. The present work was done on normal, depancreatized and adrenalectomized dogs. The experiments with adrenalectomized animals were done under nembutal anesthesia, the actual experiment following within an hour after the removal of the adrenals. The purpose of this procedure was to obtain a preparation in which no reflex secretion of epinephrin was possible but which did not suffer from adrenal cortical insufficiency. Preliminary control experiments had shown that the anesthetic used did not interfere with any of the methods of producing phosphate change in normal animals.

The amount of glucose administered throughout these experiments was 1.75 gram/kgm. body weight in a 30 per cent solution by vein. The dosage of insulin varied between 0.3 and 1 unit per kgm. body weight given subcutaneously. The dose of epinephrin was 0.1 mgm. per kgm. body weight in a 1:2000 solution, subcutaneously.

Inorganic and total acid soluble phosphates were determined by the method of Fiske and Subbarow adapted to the photoelectric colorimeter (10). Muscle hexose monophosphate was determined by the method of Cori and Cori (11). Blood sugar estimations were made by the Somogyi modification of the Shaffer-Hartman method.

RESULTS. Table 1 summarizes our data on the changes in blood inorganic phosphate and total acid soluble phosphate following the administration of glucose, insulin and epinephrin respectively to 75 dogs. It may be seen that whatever the substance administered and regardless of the type of animal used, the fall in inorganic phosphate was not accompanied by a fall in total acid soluble phosphate of the blood. The latter actually rose in most cases for reasons which we cannot explain. In a number of experiments the determination of cell volume by the hemato-

crit method failed to account for the change in total acid soluble phosphate. Under these circumstances it is evident that one cannot explain the fall in inorganic phosphate by supposing that it leaves the blood to enter the muscle, since that would necessitate a fall in total phosphate. The possibility that the inorganic phosphate actually goes into the muscle but that an equivalent or greater amount of organic phosphate enters the blood simultaneously, is discounted by such work as that of Pollack et al. (7). He found no change in the phosphate partition of perfused muscle, following the fall in inorganic phosphate in the perfusing blood. It must be concluded that an esterification of the inorganic phosphate occurred within the blood in Pollack's experiments, and presumably in ours. However, it is impossible to exclude at the present time, the pos-

TABLE 1

Milligrams per cent change in blood inorganic phosphate (P_0) and in total acid soluble phosphate (P_T)

The maximum decrease in blood inorganic phosphate (P_0) obtained with glucose in any depancreatized animal was 0.4 mgm. per cent. Hence no change in P_0 of this amount or less was considered to be significant throughout our work.

TYPE OF ANIMAL	GLUCOSE					INSULIN					EPINEPHRIN				
	Number of dogs	Decrease in P_0			Rise in P_T	Number of dogs	Decrease in P_0			Rise in P_T	Number of dogs	Decrease in P_0			Rise in P_T
		Min.	Max.	Av.	Av.		Min.	Max.	Av.	Av.		Min.	Max.	Av.	Av.
Normal	5	0.5	1.2	0.8	2.0	20	0.7	2.0	1.2	2.0	9	0.4	1.7	1.1	2.0
Depancreatized	7	0	0.4	0.2	1.0	9	1.3	2.8	1.9	1.0	14	0.6	0	0.3	3.0
Adrenalectomized	3	0.7	2.8	1.6	6.0	7	0.5	2.0	1.2	3.0	3	1.1	1.2	1.2	3.0

sibility of phosphate transfers between the blood and organs other than muscle. The crucial proof for a phosphate esterification within the red blood cell under the influence of insulin, would be the demonstration of such an effect on whole blood in vitro. A number of attempts in this direction have thus far met with no success.

Further inspection of our data in table 1 also shows that insulin caused a significant fall in inorganic phosphate in all three types of animals, while glucose and epinephrin were effective in the normal and adrenalectomized animals but not in the depancreatized ones. It is apparent that the fall in inorganic phosphate occurs only in the presence of the pancreas or of administered insulin. Since neither endogenous nor administered epinephrin need be present for the effect it may be supposed that the fall in inorganic phosphate which follows the injection of epinephrin into normal animals is a result either of a rise in the blood sugar level, which

would be equivalent to sugar administration, or to a reflex secretion of insulin consequent to the hyperglycemia.

The fact that the change in blood phosphate following glucose, insulin or epinephrin administration is due to a transformation of the inorganic phosphate to an organic form outside the muscle, leaves unexplained the previously observed simultaneous change in muscle hexose monophosphate following insulin or epinephrin administration, and the absence of this effect when glucose is given. Table 2 summarizes simultaneous observations on blood phosphate and muscle hexose monophosphate, and makes it possible to correlate the blood and muscle effects in a satisfactory manner. It may be seen that in the absence of the adrenal glands insulin did not cause a rise in muscle hexose monophosphate although it produced its

TABLE 2

Change in inorganic phosphate (P_0) and total acid soluble phosphate (P_T) of the blood, and in hexose monophosphate (HmP) of the muscle

EXPERIMENTAL CONDITIONS	DOG NUMBER	CHANGE IN BLOOD		CHANGE IN MUSCLE
		P_0	P_T	HmP*
		mgm. per cent	*mgm. per cent*	*mgm. per cent*
Depancreatized	1	−0.3	0	+9.5
Given epinephrin	2	−0.1	+3.0	+10.9
(0.1 mgm. per kgm. subcutaneous)	3	−0.4	0	+9.4
Adrenalectomized	1	−1.9	0	−0.5
Given insulin	2	−1.6	+2.0	−0.3
(0.3 unit per kgm. subcutaneous)	3	−1.2	+3.0	+0.3

* In terms of P.

usual diminution in blood inorganic phosphate. However epinephrin in the depancreatized dog, while causing no significant fall in blood inorganic phosphate, resulted in a definite increase in the muscle hexose monophosphate. It is evident that the rise in hexose monophosphate is a result of epinephrin activity within the muscle, according to its well known action in causing a breakdown of glycogen, as demonstrated in vitro by Cori (12).

It may also be of interest to record certain incidental findings in the course of this work.

a. The presence of the hypophysis is not necessary for the effects on blood phosphate. Hypophysectomized dogs exhibited significant falls in blood inorganic phosphate after dextrose, insulin and epinephrin administration respectively. The "Houssay" animal behaved like the depancreatized dog.

b. The recovery from the fall in blood inorganic phosphate caused by

any of the three agents employed, often continued to levels significantly above the initial control values. Indeed this overshooting was sometimes greater in extent than the initial decrease. This was not accompanied by a comparable increase in the total acid soluble phosphate of the blood.

c. Protamine insulin differed from regular insulin in that the parallelism between the blood sugar and phosphate curves was not maintained with the former. Figure 1 exemplifies this difference.

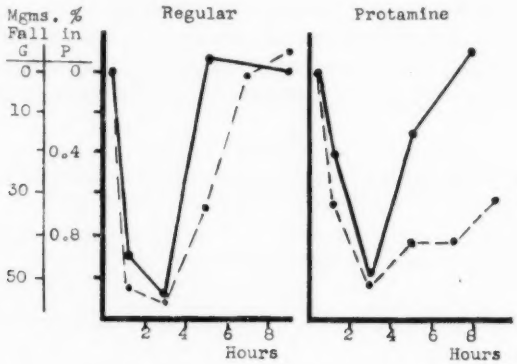

Fig. 1. Illustrating the similarity between the inorganic phosphate curves (heavy continuous lines) in normal dogs, after regular and protamine insulin respectively, despite the difference in the blood sugar curves (broken lines).

Discussion. Our results have reconciled the apparently contradictory previous evidence concerning the changes in blood and muscle phosphate following the administration of insulin, dextrose or epinephrin. It is clear that the confusion has been due to the counter-regulatory actions of the endocrine glands through which excessive insulin activity evokes a secretion of epinephrin and vice versa. When these actions are isolated by excision of the counter-regulating gland the unopposed action of the administered hormone can be observed.

In the normal intact animal epinephrin causes both a fall in the inorganic phosphate of the blood and a rise in the hexose monophosphate of the muscle. It is clear from our work that the change in the blood is not the direct result of epinephrin but is secondary to a reflex insulin secretion. In the absence of the pancreas the change in blood phosphate no longer occurs, although the muscle effect persists. Epinephrin acts directly on the muscle by stimulating the breakdown of glycogen to hexose monophosphate (12).

Insulin also causes both blood and muscle phosphate effects when administered to the normal intact animal. In this case, however, the action

of insulin on blood phosphate is direct because it occurs in the absence of the adrenal glands. The muscle effect of insulin is indirect. It does not occur in the adrenalectomized animal. The responsibility of reflexly secreted epinephrin for the muscle phosphate changes after insulin administration accounts for the absence of these changes when sufficient dextrose to prevent hypoglycemia is administered with the insulin.

The esterification of the blood inorganic phosphate by insulin must be considered in relation to the wider problem of insulin action on carbohydrate metabolism. It seems reasonable to assume that this hormone would have similar effects in tissues other than the blood, although the local situation in which it acts would be expected to alter the manner in which its action would become manifest. This conception is consistent with our previous conclusions as regards the action of insulin in muscle and liver (13, 14), to the effect that insulin catalyzes a phosphorylation which facilitates the entry of blood glucose into the cell or into a metabolic cycle preceding and necessary for both glycogen storage and oxidative breakdown.

CONCLUSIONS

1. Observations on the action of dextrose, insulin and epinephrin respectively on the blood and muscle phosphates of normal, depancreatized and adrenalectomized dogs, indicate that the phosphate changes in blood and muscle are not directly related to each other.

2. The fall in blood inorganic phosphate is due to insulin. It is not reflected in a change in the total phosphate content of the blood, and is probably due to an esterification of the inorganic phosphate outside the muscle.

3. The rise in the hexose monophosphate content of the muscle is due to epinephrin, and results from the breakdown of muscle glycogen.

4. The usual observation of phosphate changes in both blood and muscle after the administration of either insulin or epinephrin to the intact normal animal is due to the reflex evocation of the secretion of one gland by the effects of the hormone of the other gland.

5. The action of insulin in esterifying blood inorganic phosphate, is considered in relation to the general action of insulin on carbohydrate metabolism.

We wish to acknowledge the contributions to this work of Drs. M. D. Allweiss, H. Falk and L. Linn, in the several years during which this paper slowly evolved.

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HEPARIN AND NATURAL ANTIPROTHROMBIN IN RELATION TO ACTIVATION AND "ASSAY" OF PROTHROMBIN

JOHN H. FERGUSON AND ANTHONY J. GLAZKO

From the Department of Materia Medica and Therapeutics, University of Michigan

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Clotting-time (C.T.) can be a true measure of thrombin (and prothrombin) only when standardization of the test conditions includes the most rigid control of all inhibitory processes. In view of the growing importance of clinical "prothrombin" tests (15, 16) several recent publications from this laboratory (5, 7, 10) have sought to extend the experimental basis for a revised outlook on clot-inhibitory mechanisms. The present communication deals with the rôle of *antiprothrombins* (13), their occurrence in various clotting systems (including plasma), and the extent to which they may interfere with the interpretation of "prothrombin" assays.

Prothrombin assay technic. Since we are concerned particularly with the isolated first phase of clotting, the method of Quick (15) is not immediately under discussion. Our technic rather resembles the two-stage method of Warner, Brinkhous and Smith (16) except that we are chiefly interested in the underlying principles, development of which goes back to the *thrombin* assays of Fischer (4) and of Eagle (3). The essential feature of the present study is the use of a reference series of thrombin dilutions prepared in each case from the particular prothrombin being studied. This affords a means of controlling both known and doubtful variables and ensures that the clotting-time variations do have the quantitative significance attributed to them upon analysis of the experimental data.

Reagents. Routine throughout these investigations are the use of physiological *saline* (0.9 per cent NaCl) for all solutions and dilutions, and adjustment of the *pH* of all reagents to 7.5 with the aid of dilute acid (N/10 acetic) or alkali (N/10 NaOH) and the glass electrode.

The method of preparing *prothrombin*, by Howell's acetone method, from cell-free citrated dog plasma has been detailed previously (8). The *Ca salt* is N/10 $CaCl_2$. The *thromboplastin* is a saline emulsion (decanted or filtered) of frozen dog brain.

The *fibrinogen* is made from Berkefeld-filtered citrated dog plasma which has stood for several days in the ice-box. A saline solution (pH = 7.5) of the thrice precipitated [$(NH_4)_2SO_4$] protein, when tested with $CaCl_2$ and thromboplastin, shows only traces of clot after many hours, indicating the absence of all but a vestige of prothrombin.

Through the courtesy of Dr. C. H. Best, we have been supplied with purified

heparin from the Connaught Laboratories; 1 mgm. = 110 Toronto units. A 1:1000 stock solution is made in saline, and the pH adjusted to 7.5.

A crude plasma *albumin* is made by precipitating the plasma proteins between 50 and 100 per cent saturation with ammonium sulfate, dialysing away excess of salt, and obtaining a final solution in 0.9 per cent NaCl at pH = 7.5.

Tests. Incubation of thrombic mixtures at less than 10°C. favors stability and lengthens the activation period to the advantage of kinetic study. The clotting-tests proper are made by adding a measured volume of thrombic mixture, 0.5 cc., to fibrinogen, 1.0 cc., plus diluent or inhibitor (2nd-phase control), usually 0.5 cc., at 38°C. Clotting is timed from the first appearance of fibrin strands, assisted, in the prolonged clottings, by gentle agitation of the small (11 mm. diameter) tubes, held in a tilting rack in the thermostatically-controlled water bath. Test conditions are uniform with respect to volumes, temperature, pH, salt and fibrinogen concentration, and each thrombin is tested for stability by repeated C.T. determinations, extending several hours beyond the maximum activation.

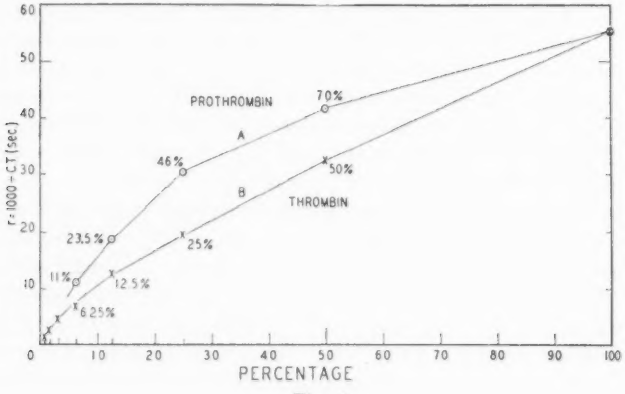

Fig. 1

DATA. Figure 1 illustrates the method of assay of prothrombin in terms of its own reference thrombin. Reciprocal clotting-times r (formulated as 1000 ÷ C. T. (seconds), Fischer, 4) are plotted against dilutions, which are designated as percentages of the original strength. Curve B is the reference thrombin. Curve A is a similar series made after the maximal activation of a corresponding set of prothrombin dilutions. A fixed amount of $CaCl_2$ and brain thromboplastin is added to each diluted prothrombin and the activation to thrombin is followed through the optimum as previously described (8). The respective thrombin "equivalents" are included in the figure. They are obtained by referring the "r" values of A to the reference curve B.

Equivalence of thrombin and prothrombin? There is a marked divergence of the data representing the two series. Obviously, dilution with saline has a different effect upon thrombin and its corresponding prothrombin.

The prothrombin series give more than the theoretical yield of effective thrombin. This has been confirmed on 16 occasions, the only differences being *a*, in two cases, more than a twofold dilution was required to bring out the discrepancy; *b*, in one experiment, only, was there coincidence of the last two or three sets of percentages, suggesting that the possibility of diluting out the divergence is rarely realized.

TABLE 1

Effects of heparin and varying thromboplastin concentration (supra-optimal) on activation of prothrombin to thrombin

pH = 7.5. Activation at 10°C. Clotting-times (sec.) at 38°C.: 0.5 cc. T + 1.0 cc. F + 0.5 cc. saline *or* heparin (C, 2)

	THROMBOPLASTIN DILUTION	INCUBATION PERIOD OF THROMBIC MIXTURE (MIN.)					
		15′	30′	60′	120′	180′	300′
A. Activation curves, without heparin							
1	1:1	16″	16″	17″	20″		
2	1:2	16″	16″	17″	20″		
3	1:4	40″	16″	16″	20″		
4	1:8	52″	16″	18″	22″		
5	1:16	63″	20″	18″	20″		
6	1:32	160″	50″	18″	19″		
B. Activation curves, with heparin							
7	1:1	80″	36″	43″	44″		
8	1:2	5°	50″	42″	44″		
9	1:4	∞	36′	43″	43″		
10	1:8	∞	5°	42″	44″		
11	1:16	∞	∞	4°	47″	42″	
12	1:32	∞	∞	∞	3°	75″	40″

C. Thrombin dilution curves

		DILUTION				
		1:1	1:2	1:4	1:8	1:16
1	Saline	17″	30″	44″	75″	126″
2	Heparin	18″	32″	65″	170″	800″

As compared with activated plasmas (defibrinated), in which the thrombin formed is very unstable (except in high dilution, 16) because of progressive (serum-) antithrombin (10), our thrombins are sufficiently stable over several hours to discount any possible rôle of thrombinolytic factors. That this is true for the weaker, as well as for the full-strength, prothrombins is borne out by the finding in the data of the subsequent experiments (table 1), which demonstrate that there is no appreciable loss of

potency when prothrombin is activated very slowly, so that any lytic factor present should have plenty of opportunity to affect the first portions of thrombin formed.

Immediate antithrombins (12) can be ruled out, since the same reagents and identical dilutions are used in both thrombin and prothrombin series. Any change in the actual clotting conditions, therefore, must be the result and not the cause of the greater thrombin yields.

By a process of exclusion of known variables, we are led to explain the phenomenon of increase in thrombin yield on prothrombin dilution as due to diminution in the effect of a hitherto unrecognized naturally-occurring *antiprothrombin*. Such an agent has been identified in similar tests on diluted (50–100×) defibrinated (56°C.) plasma. Qualitatively, at least, its action may be compared with the first phase effects of heparin (5).

Mechanism of thrombin formation and the antiprothrombic actions of heparin. Section A of table 1 shows a series of controls in which a single full-strength prothrombin is activated in the presence of various dilutions of brain thromboplastin. The close similarity of the *optimal* clotting-times (16″–18″) is good evidence of the completeness of thrombin formation in all cases. We are dealing, therefore, with a set of conditions under which the prothrombin and calcium (*cf.* 6) are fixed and the thromboplastin always in excess. The only difference between the activation data is in the *rate* of thrombin formation, which is indicated by the length of time required for maximal activation. The weaker the thromboplastin, the longer the period in question.

Section B is a repetition of the foregoing but with an added antiprothrombic factor, namely, a small fixed amount of purified heparin in each thrombic mixture. Dismissing the few seconds discrepancies due to slight thrombin instability (progressive antithrombin,, 10), the final clotting-times (circa 40″) are sufficiently uniform to indicate that the conversion, in all cases, is as complete as the new conditions will allow. The most marked action of heparin in the first phase of clotting is the retardation of the prothrombin conversion *rate*, and the clear inverse relation to thromboplastin concentration supports the conclusion that this action is *anti-thromboplastic*.

Sometimes the thrombic potency finally attained in the presence of heparin is identical with the controls (5) but often, as in the present experiments, there is a distinct lessening of optimal potency. This is clearly unrelated to the amount of thromboplastin or rate of thrombin formation. Compare the 40″ (approx.) optima in B with the 16″–18″ in A. The second phase control (C, 1) indicates a negligible effect (1″) on the thrombin-fibrinogen interaction, and there is obviously little or no second-phase cofactor ("pro-antithrombin," 14) or there would be an immediate antithrombic action in the second phase (12). It must be concluded that the 24-

second difference between A and B represents a second component in the first phase action of heparin, namely, a true *anti-prothrombic* effect, manifested by a reduction in the *amount* (effectiveness) of the thrombin formed. In the example cited, this is equivalent, approximately, to a 70 per cent loss of effectiveness.

First phase co-factor for antiprothrombic action of heparin. In contrast to Astrup (1), we always find that the antiprothrombic actions of heparin

TABLE 2

Effects of plasma "albumin" (crude) on the action of heparin in the first and second phases of clotting

Thrombic mixtures (T) = 4 cc. prothrombin + 5 cc. "albumin" (*or* saline) + 0.5 cc. (= 2.5 Toronto *units*) heparin (*or* saline) + 0.25 cc. brain thromboplastin + 0.25 cc. N/10 $CaCl_2$, kept at 7.5°C. for times (min.) stated. Clotting-times (sec.) for 0.5 cc. T + 1.0 cc. fibrinogen + 0.5 cc. saline (*1–5*) *or* 0.5 cc. of a saline mixture containing amounts of heparin, "albumin," *or* both, to make *6, 7, 8* equivalent to *2, 3, 4*, respectively. C. T.'s at 38°C. and pH = 7.5.

T	INHIBITOR	5′	15′	30′	60′	90′	120′
	A. Activation curves (first phase)						
1		95″	22″	18″	18″	18″	18″
2	Heparin	∞	*	900″	30″	23″	23″
3	Albumin	40″	20″	19″	20″	25″	29″
4	Hep. + alb.	∞	∞	∞	∞	∞	∞

B. Dilution curves (second phase controls, using T_1)

		DILUTION						
		1:1	1:2	1:4	1:8	1:16	1:32	1:64
5		18″	23″	38″	56″	97″	145″	198″
6	Heparin	19″	30″	55″	235″	*	∞	∞
7	Albumin	18″	25″	39″	61″	100″	150″	260″
8	Hep. + alb.	23″	65″	1030″	1620″	*	∞	∞

* Indicates trace of clot overnight; ∞ = no clot.

in our particular systems (5) require no *added* co-factor, but it remains an open question whether an unknown co-factor accompanies the experimental preparations. Earlier reports (cit. 2) of a first phase co-factor are unconvincing, since the experiments described are not calculated to show up early antiprothrombic action or to rule out immediate antithrombic (second phase) effects. These objections are avoided in the data exemplified in table 2.

The plasma "albumin" (see *reagents*) had no antiprothrombic action but rather a slight *thromboplastic* effect (cf. 7), shown in the early stages of

prothrombin activation. The chosen quantity of heparin had some anti-prothrombic action of its own. When the heparin and crude albumin were used in conjuction, however, a marked synergism (potentiation) occurred, with complete inhibition of thrombin formation. The second phase controls show the degree of (immediate) antithrombic action, which, for the selected amounts of the inhibitory agents and full-strength thrombin, is almost negligible in all three cases.

It is concluded that a *first phase co-factor* undoubtedly exists in crude plasma "albumin." It assists heparin to a much greater inhibition of thrombin formation than the second phase co-factor ('pro-antithrombin') contributes to the action of heparin on fully-formed thrombin.

COMMENT. In spite of demonstrated possibilities of interference by progressive antithrombin, immediate antithrombin (12), and natural antiprothrombin (v. supra), the dilution technique of the Iowa workers (2, 16) is offered as a practical method of "prothrombin" determinations. Our critical analysis is based upon a restricted and modified technique which aims not to serve the purposes, or to question the clinical usefulness, of a general method of assay, but to afford a high degree of control of the cited inhibitors in order to interpret the processes involved in any "prothrombin" method. The above data show that the theoretical basis for definition of assay standards for thrombin and prothrombin should include cognizance of these variables. In the absence of equivalence between prothrombin and thrombin dilutions, it is impossible to assay prothrombin *both* in terms of an arbitrarily fixed clotting-time *and* a definite thrombin dilution value. For practical purposes, it is suggested that at least two clotting-time reference points be selected, with a wide enough range to show up any effects due to the inhibitors mentioned. Pending answer to these suggestions, current "prothrombin" methods must be regarded as empirical and accepted with reservations.

SUMMARY

By quantitatively studying fibrinogen clotting-times with thrombin and prothrombin (maximally activated with *Ca* and thromboplastin), it is shown that the prothrombin dilutions give more than the theoretical yield of thrombin. The thromboplastin variable is ruled out, as it is optimal in all cases. Under these circumstances, thromboplastin variations merely affect the rate of thrombin formation and the optimum clotting-time is a measure of the thrombin yield.

The addition of a fixed amount of heparin slows the rate of prothrombin activation and may result in a lessened amount (effectiveness) of the thrombin formed. The first action is anti-thromboplastic, interpreted as an inhibition of the tryptase-like thromboplastic enzyme (9, 11). The second effect is truly anti-prothrombic, due to an action of prothrombin protein

itself (cf. 4). These two phenomena comprise the "antiprothrombic" (first phase inhibiting) actions of heparin and are easily separable, by control experiments, from the second phase inhibitions, (a) *progressive* (here negligible) and (b) *immediate*. An immediate antithrombin (12) is formed by heparin plus a plasma co-factor (proantithrombin). A similar, but independently acting, *co-factor for the first phase* is demonstrated in crude plasma "albumin." It markedly potentiates the antiprothrombic actions of heparin.

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QUANTITATIVE EFFECTS OF IMMEDIATE ANTITHROMBINS[1]

ANTHONY J. GLAZKO AND JOHN H. FERGUSON

From the Department of Materia Medica and Therapeutics, University of Michigan, Ann Arbor

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In a recent publication in this Journal (3), inhibitors of blood coagulation have been differentiated as follows: 1st phase, antiprothrombins (7); 2nd phase, antithrombins, (a) immediate, (b) progressive (4). This paper deals with quantitative aspects of *immediate* antithrombins, relating their concentration to the relative effectiveness of thrombin as determined by the clotting-time for a test fibrinogen, noting their rôle in thrombin assay, and raising the question of their occurrence in natural clotting systems, including plasma. It has previously been shown that polyvalent anions and also heparin (*plus* its co-factor) antagonize thrombin, whereas cations act directly on fibrinogen (5, 6). The term "immediate antithrombin," therefore, is not so much the designation of a specific thrombin-neutralizing agent as the expression of the ability of the agent to modify the fundamental physico-chemical conditions controlling the thrombin-fibrinogen interaction.

METHOD. Using a standard fibrinogen and a series of thrombin dilutions, the clotting-times (C.T.) afford values for a curve of reference, by which similar data, obtained in the presence of various immediate antithrombins, may be read off as *effective thrombin concentrations* and the degree of inhibition thus measured in terms of the amount of antithrombin used.

Technic. The stability of the thrombin (T) is controlled by keeping a portion of the solution at 38°C. and testing samples at intervals for clotting-power when mixed with fibrinogen solution (F). The clotting is timed from the addition of 1 cc. F to a mixture of 0.5 cc. T and 0.5 cc. saline (0.9 per cent NaCl). The C.T. in these controls must not alter more than a very few seconds in the course of the experiments. In the inhibition tests, the antithrombin, suitably diluted, is substituted for the saline. For the reference curve, the clotting-times of the series of thrombin dilutions are plotted against relative thrombin concentrations, expressed as percentages of the original strength.

Reagents. The citrated dog plasma used in these experiments is preserved by preliminary Berkefeld filtration, a trace of thymol, and storage at 5°C. Fibrinogen

[1] Assisted by a grant from the Horace H. Rackham Research Fund.

is prepared by three precipitations with ammonium sulfate (2). Thrombin is made by the Eagle technic (1): the CO_2-precipitate from diluted plasma is redissolved and activated with $CaCl_2$ and a saline emulsion of dog brain (thromboplastin), defibrination being effected by continual stirring with a glass rod. The thrombin is further purified by acetone precipitation (4). Solutions are made from stock by evaporating off the supernatant acetone (air-jet) and redissolving in 0.9 per cent NaCl solution. The agents to be tested are accurately weighed out and dissolved in distilled water to known volume and strength of solution. The cited concentrations (see tables) refer to amount of agent in final mixture after the addition of all the reagents. Solutions are routinely adjusted to pH=7.4 (glass electrode). Clotting tests are carried out at constant temperature (25°C.) in serological tubes (11 mm. diameter) which are tilted once a second in a mechanical rocker. The clotting is timed from the moment of mixing the thrombin and fibrinogen to the first appearance of fibrin strands (4).

Effects of ferrocyanide, etc., on thrombin clotting of fibrinogen. The immediate antithrombic action of various electrolytes and of heparin was studied for several dilutions of thrombin but a constant amount of fibrinogen. Ferrocyanide was selected as a typical anion of high valency (-4) and a series of dilutions was tested with each thrombin. No significant oxidation-reduction reactions occur under the conditions of these experiments.

In the data graphically presented in figure 1, the various concentrations of ferrocyanide were tested in relation to four different strengths of a thrombin preparation. The figure clearly shows a direct relationship between clotting-time and ferrocyanide concentration for each of the thrombins used. The approximation of the experimental results to a straight line is very satisfactory over a limited range of ferrocyanide and thrombin concentrations, although considerable deviations may occur under some experimental conditions. The *linear relationship* has been found repeatedly within a clotting-time range of about 25 to 120 seconds, i.e., with moderately weak thrombins and not too small amounts of ferrocyanide (see later). It is also evident from figure 1 that the *slopes* of the lines (a) are inversely related to the concentrations of thrombin (E).

K_2SO_4 and KCl have also been studied and found to be much less inhibitory. In using a diluted (1:4) thrombin, the inhibition due to 0.0625 M K_2SO_4 was approximately equal to that of 0.125 M KCl and to that of 0.0039 Molar $K_4Fe(CN)_6$.

Heparin. Heparin (plus its second phase co-factor, *proantithrombin*, 8) resembles ferrocyanide in its relations to fully formed thrombin. Figure 2 represents an experiment in which varying quantities of heparin were tested with four different thrombin dilutions. Plasma, diluted with saline, was used as the source of fibrinogen in order to furnish the heparin co-factor. Sufficient citrate was added to prevent activation of the prothrombin in the plasma.

The results indicate that the clotting-time for each thrombin solution is proportional to the amount of heparin present. Again, the slopes of the

inhibition curves are inversely related to the thrombin concentration. Since data with ferrocyanide (fig. 1) are just as readily obtainable with the use of similar diluted plasma, instead of purified fibrinogen, it is evident that the results of the heparin and ferrocyanide experiments are strictly comparable. The quantitatively analogous behavior affords additional

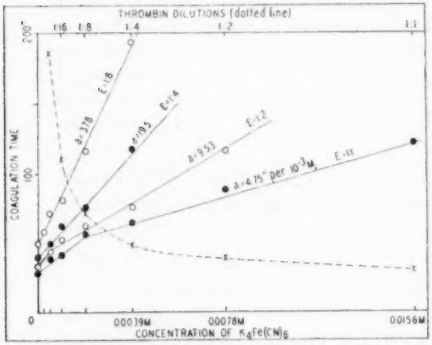

Fig. 1

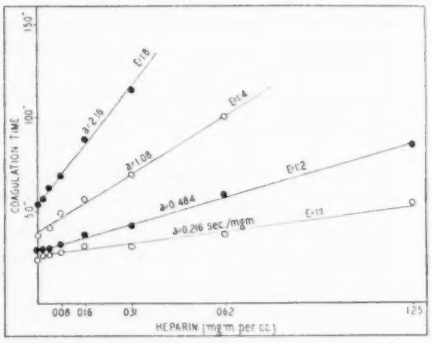

Fig. 2

Fig. 1. Effect of varying ferrocyanide concentration on clotting-times of thrombin-fibrinogen mixtures. Temp. = 25°C.; pH = 7.4. C.T. (sec.) for 1.0 cc. F + 0.5 cc. T + 0.5 cc. inhibitor. Four different thrombin concentrations (E) are tested. The dotted line is a *reference curve*, made with a series of saline dilutions of thrombin, by which clotting-times may be converted into *effective* thrombin concentration, expressed as percentage of original strength.

The linear relation between clotting-time (y) and molal concentration of ferrocyanide (M) may be expressed by the equation

$$y = a\,M + c \quad \ldots\ldots 1,$$

where a and c are constants representing, respectively, the *slope* of the "inhibition curve" and the Y-axis intercept. The inverse relation of slope to thrombin concentration (E) may be expressed by the differential equation

$$dy/dM = K/E \quad \ldots\ldots 2,$$

where K is a constant. The differential factor (dy/dM) is identical with slope a, for all values of M, when the direct relationship expressed by equation 1 holds.

Fig. 2. Effect of varying heparin concentration on clotting-times of mixtures of citrated plasma and four different thrombin concentrations. Same conditions and analysis as in figure 1.

support for the conclusion that heparin and polyvalent anions affect coagulation through a fundamentally similar mode of action (5).

Assay of thrombin and prothrombin. We have noted that the *slope* of the inhibition curve (with ferrocyanide, for instance) is an index of thrombin concentration. Although difficulties are to be anticipated in practice, this offers a new principle for thrombin, and prothrombin, assay.

Naturally-occurring immediate antithrombins in plasma. Table 1 illus-

trates tests made with a single thrombin preparation and three different coagulable solutions: (a) purified fibrinogen, (b) dog plasma I, from a healthy animal under nembutal anesthesia, (c) dog plasma II, from an animal under morphine-urethane anesthesia and in a condition of shock due to prolonged experimentation with vasomotor drugs. At the same time (expt. D) the thrombin was tested on fibrinogen in the presence of varying concentrations of potassium ferrocyanide. Although the plasmas

TABLE 1

Assay of the immediate antithrombins of plasma

	THROMBIN DILUTION									
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
A. Fibrinogen	11″	13″	16″	20″	25″	35″	52″	75″	115″	∞
B. Plasma I (1:10)	13″	16″	20″*	27″	42″	60″	87″	95″	120″	200″
C. Plasma II (1:10)	60″	55″	48″†	52″	55″	58″	80″	104″	∞	∞
D. $K_4Fe(CN)_6$	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/∞
Fibrinogen+ thrombin (1:4)	100″	70″	55″	46″†	39″	34″	31″	28″	25″	22″*

Five-tenths cubic centimeter thrombin is used to clot 1 cc. of plasma or fibrinogen, as indicated, in the presence of 0.5 cc. of the specified concentration of ferrocyanide (D) or of 0.9 per cent saline (A, B, C).

* and † indicate approximately equivalent clotting-times (see text).

TABLE 2

Anomalous effects produced by dilution and inhibitors

A. Thrombin dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Clotting-time (C.T.)	36″	15″	13″	17″	22″	40″	60″	120″
B. $K_4Fe(CN)_6$	M/∞	M/2560	M/1280	M/640	M/320	M/160		
C.T. (thrombin 1:1)	36″	32″	32″	30″	47″	240″		
C.T. (thrombin 1:2)	15″	18″	20″	24″	28″	115″		

Clotting tests: 0.5 cc. saline or ferrocyanide + 1.0 cc. diluted plasma (with added citrate) + 0.5 cc. Eagle-type thrombin.

are similarly diluted and citrated, they show a marked difference in coagulability on adding the various thrombin dilutions. A somewhat crude comparison (since some minor variables, such as fibrinogen concentration and amount of citrate (in fibrinogen), were not controlled) may be made by comparing the clotting-times with 1:4 thrombin, referring each to the ferrocyanide series, as an empirical standard of assay. This gives the finding of about 64 times as much inhibitor in plasma II as in plasma I, which may be taken as indicative of a striking increase in *immediate antithrombin* in the plasma from the animal in shock.

Anomalous clot-acceleration by "inhibitors". It is not an infrequent finding (table 2) that very small amounts of ferrocyanide shorten the clotting-time of thrombin-fibrinogen mixtures, in marked contrast to the inhibition seen with greater concentrations. The same result is obtained with many plasmas, the coagulation of which is timed from the addition of Ca-thromboplastin in the presence of weak ferrocyanide. In experiments C (table 1) and A (table 2) is seen an occasional anomaly, namely, an improvement in clotting-power on diluting a thrombin preparation. The suggested explanation in all these cases is an improvement in the physical conditions for clotting.

DISCUSSION. The linear relationship which is demonstrated between clotting-times and concentration of antithrombin is significant because it indicates that the reaction between thrombin and fibrinogen is diminished by a definite amount, which can be directly related to concentration of inhibitor. From the quantitative relations established between clotting-time, strength of thrombin, and concentration of (immediate) antithrombin, it is theoretically possible to determine any one of these variables if the other two are known. Potassium ferrocyanide is an easily reproducible standard of reference for the assay of the second phase actions of heparin and perhaps for naturally-occurring inhibitors of the same general type.

SUMMARY

A direct relation is found to exist between the clotting-time (thrombin + fibrinogen-containing fluids) and the concentration of inhibitors (electrolytes or heparin plus co-factor).

The rate of increase of clotting-time with increasing concentrations of these antithrombins, i.e., the "inhibition slope", varies inversely with the thrombin concentration.

The principles of new methods for the assay of thrombin and immediate antithrombins are outlined. Potassium ferrocyanide is suggested as a reference standard for the latter.

A clot acceleration effect produced by traces of immediate antithrombins, or, occasionally, by dilution, is observed with plasma and with some thrombin preparations.

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THE RELATIVE RESPONSES OF THE DORSAL METACARPAL, DIGITAL AND TERMINAL SKIN ARTERIES OF THE HAND IN VASOCONSTRICTOR REFLEXES[1]

ALRICK B. HERTZMAN

From the Department of Physiology, St. Louis University School of Medicine St. Louis, Missouri

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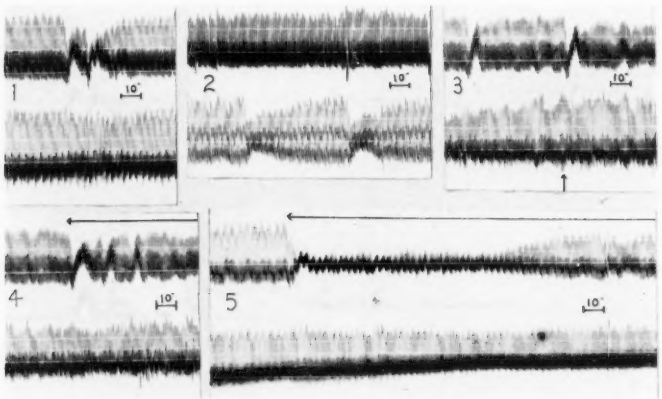
The interpretation of the effects of cold on the finger circulation indicated the advisability of extending observations on arterial reactions in the hand to include the intermediate arteries lying between the radial and ulnar arteries and the minute terminal arteries of the finger pad. The possible participation of the digital, metacarpal and volar arch arteries in the vascular reactions in the hand is not indicated in the usual experiments done on the finger or hand circulations. It is usually assumed without an adequate observational basis that the vasomotor discharges to the hand involve chiefly the terminal arterial branches since these have the most abundant innervation and, to a less extent, the larger hand arteries since these receive fewer fibers. The differences in reaction would according to this assumption be quantitative rather than qualitative. However, observations on the radial artery and the finger pad (1) where the argument applies similarly indicated that profound constriction could occur in the finger pad without effect on the radial artery. This implies a selective discharge in the vasomotor system.

Can this discharge to the hand arteries be so selective that it can involve only the terminal skin arteries without effect on the tone of the intermediate hand arteries? This paper answers the question in the affirmative.

METHOD. The reactivity of the larger arteries of the hand has been studied by recording their volume pulses with the photoelectric plethysmograph (2). The volume pulses of the dorsal metacarpal arteries and of the A. volaris indicis radialis may often be recorded with ease without much interference from the terminal skin arteries which are far less abundant on the dorsal aspects of the hand (3). Thus, when the plethysmograph is moved only slightly from the position where the artery's pulse is readily

[1] This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association. Generous assistance from the Burgess Battery Company, Freeport, Illinois, has also been received and is gratefully acknowledged.

recorded, the pulsations decrease sharply in magnitude and become difficult to record at all. This conveniently eliminates any significant error due to the pulsations at the small skin arteries. Convenient positions for the plethysmograph are at the base of the index finger on its radial aspect and in the fork between the index and middle fingers. In neither position are the plethysmograms much affected by reactions in the palmar skin. Occasionally, the first dorsal metacarpal artery (branch of the radial) lies so close to the skin surface that its pulsations are readily palpated and recorded. The digital artery pulses are usually readily recorded by placing the plethysmograph near or over the first interphalangeal joint some-



Figs. 1–5. Volume pulses of finger pad and of dorsal metacarpal artery. Time: 10 seconds.

Fig. 1. Spontaneous waves. Upper record of pad.

Fig. 2. Spontaneous waves. Raynaud's disease. Lower record of pad.

Fig. 3. First constriction is spontaneous. Second constriction is in response to a loud noise. Upper record of pad.

Fig. 4. Immersion of opposite hand in ice water. Upper record of pad.

Fig. 5. Application of cold to finger whose pad pulse is recorded in upper record.

what toward the palmar surface. Occasionally the digital artery records are mixed with effects from the terminal arteries in the pad. When this is the case, mild vasoconstrictor stimuli *seem* to elicit constrictions in the digital artery as well as in the pad. A slight shift in the position of the plethysmograph will eliminate these responses and also result in a large increase in the amplitude of the recorded pulse.

Subjects (healthy adults unless otherwise noted) were in a sitting position with the arm flexed and raised slightly. This position seems to provide the best protection against gross artifacts due to movements.

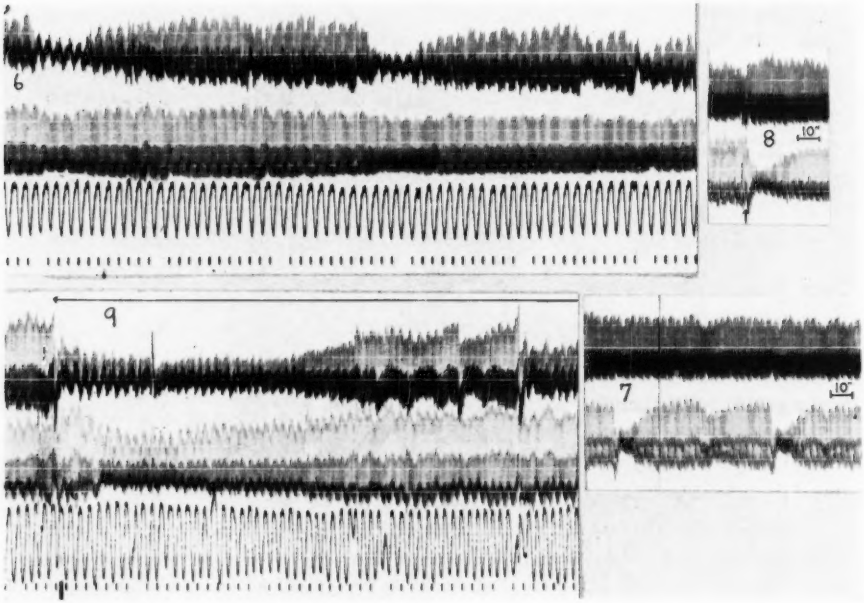
Results. The dorsal metacarpal arteries are smaller and better muscled

tubes than the radial and ulnar arteries and their immediate branches. One might therefore expect the dorsal metacarpal arteries to participate more frequently in the vasoconstrictor reflexes involving the small arteries and arterioles of the finger skin but such has not been our experience (figs. 1–5). This is particularly true when the vasoconstrictor discharges are brief as in the spontaneous waves of short duration (figs. 1 and 2), where complete absence of constrictor effect on the metacarpal arteries is usually the case. It was interesting to note that this was also true in similar observations on a case of Raynaud's disease (fig. 2). Occasionally more prolonged discharges may slightly affect the metacarpal artery pulse. The vasoconstrictor reflexes elicited by loud noises (fig. 3), by deep breaths, by immersion of the opposite hand in ice water (fig. 4) were usually without effect on the amplitude of the metacarpal artery pulses. A well sustained constriction may be elicited in the finger by local cold to it without any indication of a constrictor effect on the supplying metacarpal artery (fig. 5). The cold stimulus regularly elicits constriction in the unchilled finger as well, so the failure of the metacarpal artery to constrict must in this case be due to the selective character of the vasoconstrictor discharge. The usual absence of vasoconstrictor reflexes in the metacarpal arteries parallels the observations on the radial artery (1). In a few instances where a moderate participation of the metacarpal arteries in the vasoconstrictor discharges seemed to occur, the plethysmograph was placed at the fork of the second and third fingers and directed towards the palmar skin. One could not then be quite certain that the volume pulse record had not been directly affected by reactions of the small arteries and arterioles in the trans-illuminated skin.

The results on the digital arteries were somewhat surprising when considered from the anatomical viewpoint. These vessels have a fairly thick media, they receive a vasomotor innervation and they can go into powerful spasms (4). In view of the extreme lability of the finger circulation, the ease with which vasomotor reflexes can be demonstrated here, and the extraordinarily large changes which can and do occur in the finger's blood flow (5), one may expect vasoconstrictor reflexes to involve the digital arteries as well as the minute vessels of the pad. Only quantitative but not qualitative differences in the responses of these finger vessels may be expected if Schretzenmayr's thesis of synergistic participation of the larger arteries in the vasomotor reflexes (6) is ordinarily applicable to man. No better test of this idea could be made than in the extremely labile vascular field of the finger.

The results (figs. 6–9) indicate that the digital arteries are not usually involved in the vasoconstrictor discharges to the finger. Thus, spontaneous waves (figs. 6 and 7) and the reactions in the pad vessels to such stimuli as loud noises (fig. 8) may be and usually are without any constric-

tor effect on the digital artery. In instances of apparent participation in such reflexes one is justified in questioning the validity of the observations since exact placement of the plethysmograph is required if the pad pulses are not to affect the digital artery records. The small vessels of the pads of the three phalanges participate regularly in the vasomotor reactions of



Figs. 6–9. Volume pulses of finger pad and of digital artery at first interphalangeal joint.

Fig. 6. Spontaneous waves. Upper record of pad. Respiration and time in 5 seconds.

Fig. 7. Spontaneous waves. Lower record of pad.

Fig. 8. Loud noise. Lower record of pad.

Fig. 9. Application of cold to finger whose pad pulses and digital artery pulses are being recorded. Upper record of pad. Respiration and time in 5 seconds.

the terminal phalanx. We have seen no exceptions to this statement in healthy subjects.

The application of cold to the finger in which the reactions of the pad vessels and of the digital artery are being recorded should be an excellent test of the participation of the latter in vasoconstrictor reflexes in the finger. When this is done by methods which will be described elsewhere, the digital artery may fail to constrict until its temperature has fallen

(fig. 9). The initial constriction in the pad of the chilled finger is due to vasoconstrictor reflexes elicited by the cold and acting on the warm fingers of both hands as well as on the chilled finger. (The detailed description of these responses will appear in a following paper.) The digital artery volume pulses may increase at the moment constriction occurs in the pad. This may be the result of a rise in pressure in the digital artery due to constriction in the arterioles which it supplies. As chilling proceeds, the digital artery volume pulses may decrease somewhat (fig. 9), due possibly to a direct constrictor effect of cold on the artery.

COMMENT. These experiments are in agreement with the generally held opinion that the vasomotor reflexes act principally on the arterial gateway, the small arteries and arterioles. They also follow logically from the previously published observations on the reactions of the radial artery in similar circumstances (1) and they add evidence in support of the position taken there.

There is nothing in the known anatomy of the sympathetic nervous system and of the hand arteries which would lead one to expect that a vasomotor reflex to the arterioles and small arteries of the skin would be limited to these small vessels and would not also involve the larger arteries of the hand. Failure of the latter vessels to be included in the vasoconstrictor reflexes in the hand circulation can not be due to lack of muscle in the walls of these arteries or to the absence of an innervation. Quantitative differences between the responses of the digital arteries and those of the smaller arteries and arterioles which they supply may be expected but complete absence of a vasoconstrictor response in the digital artery at the time that its minute branches constrict powerfully is another matter. It should be emphasized that this may happen at the time that the vasoconstrictor discharges are sufficiently widespread to involve fingers and toes.

The simplest position is to consider these results as further evidence that the vasomotor reflexes are highly selective with respect to the vascular topography involved in the reactions, that these ordinary vasomotor activities are not the result of mass actions by the sympathetic nervous system. We so interpret the data of this paper.

There is nothing implied in this interpretation which would in any way negate the possibility of the larger hand arteries and the larger main arterial trunks of the arm of participating in more massive disturbances of the arterial tree than those described here.

SUMMARY

The participation of the intermediate hand arteries, the dorsal metacarpal arteries and the digital arteries, in the vasoconstrictor reflexes of the hand, has been studied by recording their volume pulses with the photoelectric plethysmograph.

These arteries do not usually participate in the so-called spontaneous waves (figs. 1, 2, 6 and 7), in the vasoconstrictor reflexes elicited by loud noises (figs. 3 and 8), by immersion of opposite hand in ice water (fig. 4), or by application of cold to the finger whose pad pulses are being recorded (figs. 5 and 9).

These results are most simply explained by considering the vasomotor reflexes as highly selective with respect to the vascular topography involved in the reactions.

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THE CIRCULATORY RESPONSES OF CHRONIC SPINAL DOGS TO ETHER ANESTHESIA[1]

WALTER S. ROOT AND FERDINAND F. McALLISTER

From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York City

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The fall in mean arterial blood pressure which occurs when ether is administered to sympathectomized dogs (1) suggests that during ether anesthesia the activity of the sympathetic nervous system is essential for the maintenance of the normal blood pressure level. Since it is known that the isolated spinal cord can mediate some sympathetic activity in response to various types of stimuli (2, 3, 4), it seemed of considerable interest to compare the circulatory responses to ether inhalation shown by chronic spinal dogs with those exhibited by sympathectomized dogs.

METHODS. The data reported in this study have been gathered from experiments on seventeen spinal dogs. All operations were performed aseptically under nembutal anesthesia. The animals were used for the ether experiments four to fifty-three days after transection of the spinal cord, the duration of the postoperative period not significantly altering the results. At the time of experimentation, the animals were in good condition. In every instance the level of the cord section was ascertained by autopsy. The sites of the spinal cord sections are shown in table 1.

The mean arterial blood pressure was determined in the femoral artery by the insertion of a glass cannula connected to a mercury manometer. The heart rates were counted from the kymograph records and checked against rates counted with a stethoscope. Blood ether concentration was measured by Ruigh's modification of the iodine pentoxide method (5). Two cubic centimeters of jugular vein blood were used for each determination.

After a control period of from one-half to one and one-half hours of rest on the animal board, the dogs were etherized by the drop method for one hour.

RESULTS. As in the experiments with sympathectomized dogs (1), only the stage of excitement and the stage of surgical anesthesia were recognized. The events occur so quickly in the dog that it is impractical to distinguish the first and fourth stages.

[1] A preliminary report of this work was presented before the American Physiological Society, Toronto, This Journal **126**: P613, 1939.

Dogs with spinal cord section below T-10. In animals with spinal cord section below T-10 (table 1), the effects of etherization on blood pressure and heart rate were the same as in normal animals (1). Figure 1 shows a blood pressure tracing from one of these animals.

Dogs with spinal cord section above T-7. In thirteen experiments on nine dogs with cord section above T-7 (table 1), the control values for ar-

TABLE 1

The distribution of the spinal cord sections

NUMBER OF ANIMALS	LEVEL OF CORD SECTION
1	7th to 8th C
2	8th C to 1st T
8	1st to 2nd T
2	4th to 5th T
1	6th to 7th T
1	11th to 12th T
1	13th T to 1st L
1	1st to 2nd L

Fig. 1. The effect of ether inhalation upon the blood pressure and heart rate of a dog six days after the spinal cord had been sectioned between T-11 and T-12.

terial pressure were slightly below normal (average 95 mm. Hg), but the resting heart rates fell within the normal range (80 to 100). In striking contrast with the effects on normal and low spinal dogs, the induction of anesthesia in high spinal animals (fig. 2) caused in every instance a precipitous fall of 30 to 60 mm. Hg in arterial blood pressure accompanied by bradycardia. As the surgical stage was entered, the blood pressure showed

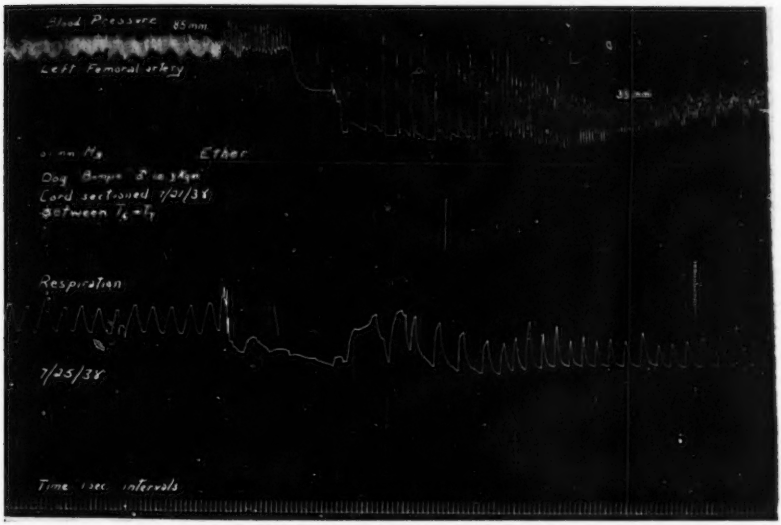

Fig. 2. The effect of ether inhalation upon the blood pressure and heart rate of a dog four days after transection of the spinal cord between T-6 and T-7.

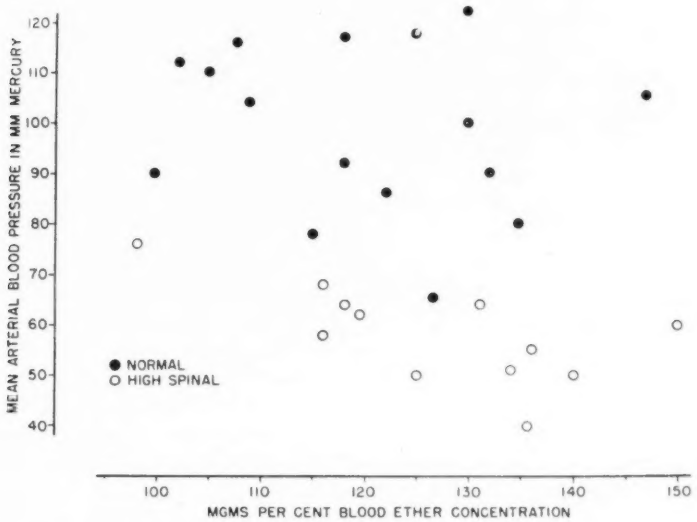

Fig. 3. The relation of the mean arterial blood pressure to the blood ether concentration in normal and high spinal dogs.

only a slight tendency to return to the control value. During full surgical anesthesia, the mean blood pressure levels usually ranged between 40 and 65 mm. Hg (fig. 3). Under these conditions the acceleration in heart rate varied with the level of cord transection. Thus, with sections at or above T-1, the rate increased to only about 135 beats per minute (the denervated heart rate), whereas with sections below T-4 the rate increased to approximately 200, as in normal animals.

The vagi were cut in three dogs with spinal cord sections between C-6 and T-1. In these animals, the administration of ether caused an abrupt fall in blood pressure without bradycardia (fig. 4). This response closely resembles that shown by sympathectomized, vagotomized dogs (1).

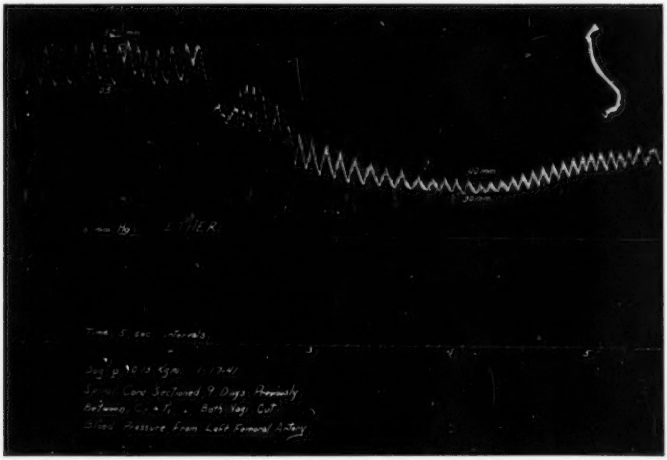

Fig. 4. The effect of ether inhalation upon the blood pressure of a bilaterally vagotomized dog nine days after the spinal cord had been cut between C-7 and T-1.

During surgical anesthesia, the blood pressures and the heart rates of vagotomized, high spinal dogs were the same as those shown by high spinal dogs with intact vagi.

The effect of ether anesthesia upon the circulation has also been studied in spinal cats. Etherization of four cats with spinal cord sections above the eighth thoracic segment caused the same blood pressure and heart rate responses that were observed in the high spinal dogs. In two cats with cord section below the ninth thoracic segment, the administration of ether produced circulatory changes identical with those shown by normal cats and dogs.

Discussion. During the excitement stage of ether anesthesia, spinal dogs exhibit the bradycardia shown by normal and sympathectomized

dogs. This response, which is absent in vagotomized, spinal dogs, is attributed to the reflex stimulation of the vagal centers by the irritant action of ether upon the respiratory mucosa (6).

During the surgical stage of ether anesthesia (blood ether, 100 to 150 mgm. per cent), the heart rate depends upon the level of the spinal cord transection. As in normal dogs, the heart rate of animals which have suffered section of the cord below the fourth thoracic segment increases to about 200 beats per minute. But if the upper thoracic segments are cut off from suprasegmental control, less cardiac acceleration is produced by surgical ether anesthesia. In dogs with low cervical transections, the heart rate increases during surgical anesthesia to about 135 beats per minute. This is the rate of the denervated heart and indicates that the rate has increased only to the extent that can be accounted for by the removal of vagal inhibition. When the vagal effects are eliminated by bilateral vagotomy, the induction of ether anesthesia causes no change in heart rate (fig. 4). These findings indicate that the isolated sympathetic cardioaccelerator centers of the spinal cord are not excited by ether.

The rise in blood pressure shown by normal dogs during the excitement stage of ether anesthesia (1) does not occur when the suprasegmental control of the spinal sympathetic centers below the sixth thoracic segment is cut off by spinal cord section at or above that level (see figs. 1 and 2). Instead, these high spinal animals show a fall in pressure similar to that exhibited by sympathectomized dogs (1). On the other hand, the administration of ether to animals with cord transections below the tenth thoracic segment produces the rise in blood pressure observed in normal dogs (fig. 1). The same relations prevail under surgical ether anesthesia. With cord sections above the seventh thoracic segment the blood pressure is low (30–60 mm.), whereas, with cord sections below the tenth thoracic segment, the blood pressure remains within the normal range (100–120 mm.) (see figs. 1 and 2). Thus, it is apparent that an important sympathetic outflow for the nervous control of blood pressure during ether anesthesia lies between the sixth and the eleventh thoracic segments.

The different vascular responses shown by high and low spinal dogs suggest that the splanchnic nerves may be of major importance in the maintenance of the normal blood pressure. In view of this, bilateral subdiaphragmatic splanchnic nerve section was performed on two dogs. The three experiments on these animals showed that ether anesthesia produced blood pressure and heart rate changes identical with those of normal animals except that the rise in pressure during the excitement stage was less pronounced. Hence, it seems that in the absence of the splanchnic nerves impulses mediated by other sympathetic vasoconstrictor fibers are able to maintain the vascular tone.

Since this study does not include measurements of the cardiac output,

an accurate evaluation of the blood pressure changes is not possible. Nevertheless, the direct relation between blood pressure variations and the amount of the sympathetic nervous system which remains connected with the suprasegmental centers suggests that the observed effects result from alterations in peripheral resistance rather than from primary changes in cardiac output. Assuming that the mechanism of peripheral vascular reactions is identical in etherized, high spinal dogs and in etherized, sympathectomized dogs, vascular dilatation may be produced either by stimulation of dilator mechanisms in the cord or by the direct action of ether or certain metabolites upon the blood vessels. In the normal animal, such dilatation is doubtless masked by sympathetic vasoconstrictor impulses originating above the cord.

SUMMARY

1. The administration of ether to dogs with the spinal cord sectioned below the tenth thoracic segment produces the same changes in blood pressure and heart rate that are shown by normal animals (fig. 1).

2. The inhalation of ether by dogs with the spinal cord cut above the seventh thoracic segment results in an immediate fall in blood pressure. This is associated with a marked bradycardia (fig. 2). During surgical anesthesia the mean blood pressure ranges between 40 and 65 mm. Hg (fig. 3). In this stage the degree of cardiac acceleration is related to the amount of the residual cardio-accelerator outflow which remains connected with suprasegmental centers.

3. The administration of ether to vagotomized, high spinal dogs (cord section C-6 to T-1) causes no change in heart rate and produces the same blood pressure responses that are shown by high spinal dogs with intact vagi (fig. 4).

4. Etherization of dogs with bilateral splanchnic nerve section produces in general the circulatory responses observed in normal dogs.

5. It is concluded that for the maintenance of normal blood pressure during ether anesthesia, the suprasegmental control of the sympathetic nervous system must extend below the sixth thoracic segment.

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THE EFFECT OF DOSAGE AND DURATION OF ADMINISTRATION ON THE ANTI-UREMIC EFFECT OF DESOXYCORTICOSTERONE

CHRISTIANE DOSNE

From the Department of Anatomy, McGill University, Montreal, Canada

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Selye (1) and Ludden et al. (2) reported that desoxycorticosterone acetate (D.C.A.) has a renotropic action which is very similar to that of testosterone and progesterone. This kidney-stimulating effect has been proven to be direct and not mediated by the hypophysis since all these steroids are active in the absence of the pituitary (3). However, since they are not as active as in the intact animal and fail to restore the atrophied kidneys of the hypophysectomized rat to their normal size, Selye concluded that most probably the kidney is dependent for the maintenance of its normal structure on the balanced production of other hypophyseal principles (growth and thyrotropic?) in addition to the luteinizing and adrenotropic hormones. Selye (4) has observed a beneficial action of both testosterone and D.C.A. on the kidney after sublimate poisoning; but even more remarkable was the constant protective effect obtained with D.C.A. in experimental uremia produced by complete nephrectomy since this action could not merely be due to the renotropic effect of the steroid (5). Furthermore, Selye and Nielsen (6) reported that D.C.A. not only prolongs the survival time and delays the clinical signs of uremia, but actually inhibits the rise in the non-protein nitrogen content of the blood after complete nephrectomy. Since several investigators have already discussed the possibility that because of their renotropic effect certain steroids may be useful in the clinical treatment of kidney disorders, this action of D.C.A. appears to be particularly important. The use of this substance in place of androgens has the definite advantage of eliminating specific effects on the sex organs. Therefore it was deemed worthwhile to investigate what would be the optimum length of pretreatment and the optimum dose for the experimental study of the antiuremic properties of D.C.A. in mice and rats.

In order to establish these conditions we performed a number of experiments on female albino mice with body weights averaging 18 grams. In the first experiment we attempted to establish the optimum daily dose, and, as shown in the table, groups 1 to 6 received subcutaneous injections

of D.C.A. dissolved in 0.2 cc. of peanut oil in doses ranging from 0.1 to 5.0 mgm. The right kidney was removed after careful decapsulation so as to prevent any injury to the adrenal and 24 hours later pretreatment was started. Six days having been tentatively chosen for the length of pretreatment, the left kidney was removed at the end of that period and the survival time of each animal was recorded. The results summarized in table 1 indicate that all doses above 0.1 mgm. significantly increase the

TABLE 1

Survival time of nephrectomized mice and rats after pretreatment with D.C.A.

	GROUP	DAILY TREATMENT	NUMBER OF DAYS	NUMBER OF ANIMALS	MEAN BODY WEIGHT	SURVIVAL TIME		P*
						Mean	Range	
					grams	*hours*		
I	1	0.2 cc. oil	6	9 mice	17	26	20-36	
	2	0.1 mgm. DCA	6	10 mice	18	32	24-38	0.06
	3	0.5 mgm. DCA	6	10 mice	17	34	23-50	0.05
	4	1.0 mgm. DCA	6	10 mice	17	35	22-50	0.05
	5	2.0 mgm. DCA	6	10 mice	17	35	22-46	0.03
	6	5.0 mgm. DCA	6	10 mice	18	34	23-48	0.05
II	7	0.1 cc. oil	6	10 mice	17	25	19-41	
	8	2.0 mgm. DCA	1	12 mice	18	28	19-45	0.40
	9	2.0 mgm. DCA	3	11 mice	17	43	35-46	<0.01
	10	2.0 mgm. DCA	6	11 mice	19	34	22-47	0.03
	11	2.0 mgm. DCA	12	10 mice	19	36	22-48	0.02
	12	2.0 mgm. DCA	24	10 mice	19	38	23-47	0.02
III	13	0.1 cc. oil	3	12 mice	17	30	24-40	
	14	2.0 mgm. DCA	3	12 mice	18	48	40-56	<0.01
	15	2.0 mgm. DCA	6	12 mice	18	38	26-51	0.01
IV	16	0.2 cc. oil	3	12 rats	90	33	29-39	
	17	2.0 mgm. DCA	3	12 rats	96	36	31-42	0.05
	18	5.0 mgm. DCA	3	12 rats	95	41	33-52	<0.01

* Probability calculated from Fisher's table of t (7). Results are considered to vary significantly from the normal when P is equal to or smaller than 0.05.

survival time, and the optimum daily dose of D.C.A. in mice is 2.0 mgm. since group 5 has the lowest P value combined with the longest mean survival time.

Having obtained an approximate optimum daily dose of D.C.A., we performed a second experiment (groups 7 to 12) in which all animals were treated with the dose of 2.0 mgm. and the length of pretreatment varied from 1 to 24 days. In all other respects the experiment was conducted as in the previous series, the right kidney being removed 24 hours before the

first injection and the left kidney on the last day of pretreatment. Here we found a significant difference in the length of survival of the different groups wherever the number of days of pretreatment was at least 3, with an optimum value for 3 days where P was less than 0.01 and the mean survival time longest.

A third similar experiment (groups 13 to 15) emphasized these results giving as the optimum daily dose for mice 2.0 mgm. of D.C.A. and as the optimum length of pretreatment 3 days.

After having ascertained optimum conditions in the mouse, we attempted to do the same in the rat. For this purpose we performed a fourth experiment (groups 16 to 18) on 36 albino rats with 6 males and 6 females in each group, all having an average weight of 95 grams. All groups were pretreated for 3 days, one with 0.2 cc. of peanut oil, one with 2.0 mgm. and the others with 5.0 mgm. of D.C.A. daily subcutaneously. The best among the doses tested was the 5 mgm. daily dose with 3 days' pretreatment.

SUMMARY

Desoxycorticosterone acetate exerts a protective effect in experimental uremia produced by complete nephrectomy and although it does so when given in ranging doses and for different periods of time, the optimum conditions for the study of these antiuremic properties have been found to be 2.0 mgm./day in the 18 gram mouse, and 5 mgm./day in the 95 gram rat given subcutaneously in oil for 3 days.

Acknowledgments. The expenses of this investigation have been defrayed by a grant of the United States National Research Council Committee on Research in Endocrinology administered by Dr. Hans Selye. The author is greatly indebted to Drs. Gregory Stragnell and Erwin Schwenk of the Schering Corporation of Bloomfield, New Jersey, for the desoxycorticosterone acetate used in these experiments.

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VARIATIONS IN FILLING AND OUTPUT OF THE VENTRICLES WITH THE PHASES OF RESPIRATION

T. E. BOYD AND MARY C. PATRAS

From the Department of Physiology and Pharmacology, Loyola University School of Medicine, Chicago

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Several workers have made use of the cardiometer for studying respiratory effects on ventricular filling and output. The application of their findings to normal breathing is, however, somewhat questionable. Henderson and Barringer (1913b) used dogs with bilateral open pneumothorax. The lungs were kept inflated by a stream of air under positive pressure, controlled by a Müller water valve. The rate of insufflation was so adjusted that the animal continued to make rhythmic respiratory movements, but the intrathoracic pressure was at least atmospheric and the intrapulmonary pressure somewhat higher. Eyster and Hicks (1933) and Cahoon, Johnson and Michael (1940), after applying the cardiometer, closed the chest so that natural breathing could go on; but the cardiometer was connected to a recording device moving in outside air. The ventricles thus had to fill against an unvarying atmospheric pressure. Eyster and Hicks recognized this as a possible source of error, but did not consider it to be of critical importance. The ventricles, because of their thick walls, were believed to be little if at all affected by the normal respiratory changes of external pressure upon them. This assumption is not uncommon in the earlier literature, but it seems always to have lacked experimental support, and is shown below to be untenable. In the present study we have used a recording system in which external pressure on the ventricles is always approximately equal to intrathoracic pressure, varying as the latter does with the phases of respiration.

APPARATUS. The arrangement is shown diagrammatically in figure 1A. The recording device is a tambour with a wide membrane, inclosed on both sides. The air chamber on one side is connected to the cardiometer, that on the other to the intrapleural space of the thorax. Except for these connections the chest is closed, and the animal breathes naturally. The side outlets at C_1 and C_3 are shut off by clamps after initial adjustment of the air content on both sides of the membrane. On the cardiometer side, the volume should be so regulated that the excursions to be recorded are approximately equal in both directions from the plane position of the

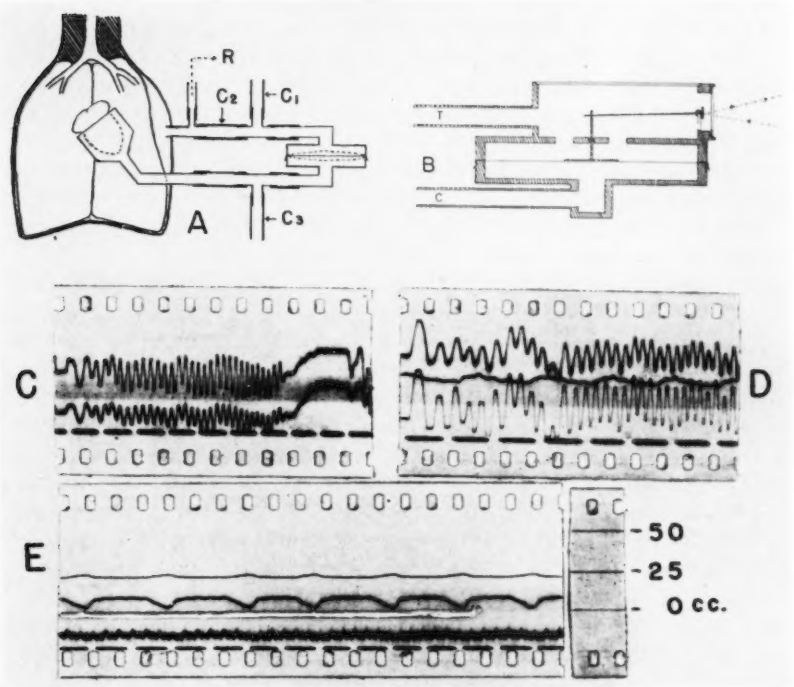

Fig. 1. A. Diagram of recording system. *R*, outlet to segment capsule recording respiration. C_1, C_3, side outlets which are clamped off after initial adjustment of air volume on both sides of the tambour membrane; C_2, alternative position for clamp at C_1. Further explanation in text.

B. Recording tambour. Diameter of membrane 12 cm., walls permit maximum excursion of 1 cm. in each direction. Inside dimensions of box above (shown in longitudinal section) 3 x 3 x 9 cm. The lid is of glass, removable but sealed by modelling clay. *T*, outlet to intrapleural space; *C*, outlet to cardiometer.

C. Response of tambour (above) to moving piston of syringe (below) upper chamber open to outside air. Time, seconds.

D. Response of tambour (below) to moving piston of syringe (above). The upper chamber was connected to the intrapleural space of a dog breathing naturally. The middle record is of intrapleural pressure.

E. From above down: respiratory displacement of tambour, volume scale shown at right; intrapleural pressure, reduced by about 8 cm. H_2O at each inspiration; arterial pressure; time, seconds. The lower chamber of the tambour was connected to a flask with a fixed air space of 100 cc.

membrane. When the upper chamber is first connected to the thorax, air is withdrawn through C_1 to inflate the lungs; but enough should be left to maintain a small pneumothorax at all times, allowing free movement

of air to equalize pressures between the tambour and the intrapleural space. As long as this condition is observed the ventricles fill, during diastole, against the existing intrapleural pressure, plus the slight resistance of the membrane itself. The membrane is thereby displaced upward, and a corresponding volume of air driven over into the thoracic cavity. This movement is reversed at systole. Ventricular volume changes are recorded as large and rapid excursions, superimposed on slower and much smaller respiratory waves.

Details of the tambour are shown in figure 1B. The ordinary lever arrangement obviously cannot be used for recording, on a kymograph in outside air, the excursions of a membrane with subatmospheric pressure on both sides. The membrane here used carries a light disc supporting a flat vertical shaft, both of thin aluminum. The shaft, projecting into the boxlike superstructure, engages a short lever with a mirror pivoted at its fulcrum. The glass window, transmitting the light beam to and from the mirror, is a convex lens, focusing the beam on a ground-glass plate at a distance of 30 to 40 cm. The excursions, too large for direct projection into the camera, are recorded by a camera with an ordinary lens, placed beyond the ground-glass plate. The bulk of the tambour interferes with the simultaneous projection of other light beams, except from an equally short distance. We therefore had to use, for arterial and venous pressures, manometers of relatively high sensitivity and low frequency. Arterial pressure was recorded from the carotid. A catheter was passed down the external jugular vein to the right auricle. It was connected to a manometer so damped that the rapid pulsations in the cardiac cycle were nearly suppressed, the slower respiratory waves of venous pressure being recorded. The arrangement has the advantage of allowing continuous observation of the moving spots on the ground-glass plate.

We have used in the tambour membranes of varying sensitivity. Each was tested by connecting, to the lower chamber, a water manometer and a large syringe containing 100 cc. of air (a volume approximately equal to the average air space around the ventricles in the cardiometer). The piston of the syringe was then moved slowly over a volume range of 50 cc., 25 cc. in each direction from the original position. Between these limits, the pressure change registered with different membranes was 4 mm. H_2O, for the slack type described by Henderson and Barringer (1913a); 24 mm., for medium-weight rubber dam under considerable tension; and 10 mm., for light dam under tension just sufficient to prevent sagging. The results described below were obtained with all three membranes; but we prefer the third type, which was used for all the graphic records shown in the figures. It offers the minimum resistance to ventricular filling, consistent with stability of the membrane when the tambour is placed on its side. This position is most convenient, since it allows horizontal excursions of the beam for recording on film moving vertically.

All the membranes used operate under such low pressures that they probably cannot follow in detail the rapid changes of ventricular volume (Wiggers and Katz, 1922). They appear to be capable, however, of revealing any gross variations of diastolic or stroke volume. To test this, the lower chamber was again connected to the syringe, and the piston moved at varying rates, up to 270 per minute. To the piston was attached a long lever, with a mirror pivoted at the fulcrum for optical recording. The membrane follows the displacement of the piston with fair accuracy. This is true whether the upper chamber of the tambour is left open to outside air (fig. 1C) or is connected to the intrapleural space of an animal breathing naturally (fig. 1D).

It may be pointed out that in figure 1D the movements of the piston were not synchronized with the cardiac cycle, yet they caused only minute changes of intrapleural pressure. Volume changes in the syringe and tambour must therefore have been compensated by a nearly equal movement of air in and out through the trachea, the lungs acting as a second sensitive membrane in series with that of the tambour. In cardiometric recording, with the arrangement shown in figure 1A, volume changes of the ventricles do not have to be so compensated by equal changes of air volume in the lungs. Each change of ventricular volume is accompanied by an opposite change in the volume of blood held in other thoracic vessels; and this redistribution of blood in the cardiac cycle largely compensates for the movement of air back and forth between tambour and thorax. The air drawn into the trachea at systole, and expelled at diastole, needs only to equal the simultaneous change of blood volume in the thorax as a whole. Calculations of the latter are not in entire agreement (Hamilton, 1930; Blair and Wedd, 1939); but according to the highest estimate it amounts only to a fraction of the stroke output from one ventricle.

Under the conditions diagrammed in figure 1A, the membrane will be displaced to some extent by respiratory movements. This effect is of small magnitude. Assuming the resistance of the membrane to be negligible, the air in the entire system behaves as if it were held in a closed pneumothorax, expanding with inspiration in direct proportion to the reduction of intrathoracic pressure. The total air volume of the system, including the variable space of the cardiometer but not the actual pneumothorax, is approximately 460 cc., of which about 240 cc. is on the cardiometer side of the membrane. Supposing intrapleural pressure to change from 755 mm. Hg at expiration to 750 mm. at inspiration, the displacement of the membrane in each respiratory cycle would be 5/755 x 240, or less than 2 cc. With deeper inspiration the respiratory displacement is proportionally greater, but it is always small in comparison to the stroke output from the two ventricles of a large dog. The respiratory effect may be shown in uncomplicated form by connecting the upper chamber of the

tambour to the intrapleural space in the usual manner, and the lower chamber to a flask with a fixed air space of 100 cc. (fig. 1E).

The respiratory changes of intrapleural pressure are effectively transmitted to the cardiometer. This is shown by the fact that respiration can be recorded by a segment capsule connected either to the upper or to the lower chamber, without appreciable difference in the magnitude of the excursions. Any displacement of the tambour membrane from its plane position implies, of course, a pressure difference on the two sides; but the respiratory displacement involves a pressure difference of the order of 1 mm. H_2O, negligibly small in comparison with the respiratory changes of intrathoracic pressure. Ordinarily we recorded respiration by connecting the segment capsule to the outlet at *R* in figure 1A. It was then possible to shift pressure on the ventricles from intrathoracic to atmospheric, or vice versa, without disturbing the respiratory record. A clamp may be applied at C_2 (fig. 1A) and the clamp at C_1 simultaneously released, opening the upper chamber of the tambour to outside air. This reproduces essentially the conditions existing in the experiments of Eyster and Hicks and of Cahoon, Johnson and Michael, referred to above. For comparison we have in each experiment placed the ventricles alternately under intrathoracic and under atmospheric pressure.

The actual pneumothorax required for proper operation of the system is not enough to embarrass the respiration seriously. Our experiments have been continued for three hours or longer, the animals remaining in good condition without artificial respiration. The dogs used were large, weighing from 14 to 22 kgm. They were anesthetized by barbital-sodium, 0.3 gram per kgm., given intraperitoneally. After tracheotomy, the chest was opened by resecting one rib for the greater part of its length. A cardiometer of suitable size was fitted over the ventricles and the chest closed. Connections to the tambour were provided for by two metal tubes of 9 mm. bore, left in the incision. One led to the cardiometer, the other opened into the intrapleural space.

Results. During complete respiratory standstill, induced by weak central stimulation of one sectioned vagus, diastolic volume and stroke output remain for a time nearly constant (fig. 2A). If the apnea is prolonged, there is a very gradual increase of both, accompanied by a rise of arterial pressure, and, if the second vagus is intact, by a moderate slowing of heart rate.

When the animal is breathing, the level of the entire cardiometer record rises with each inspiration. The rise is due, in part, to inspiratory expansion of air in the system and the resulting upward displacement of the tambour; but this effect is relatively small and does not continue into the expiratory pause (compare fig. 2D, first part, with fig. 1E). When due allowance is made for it, there is still an evident inspiratory increase of

ventricular volume, both at the end of diastole and at the end of systole. There is also an augmentation of stroke output. These effects are minimal

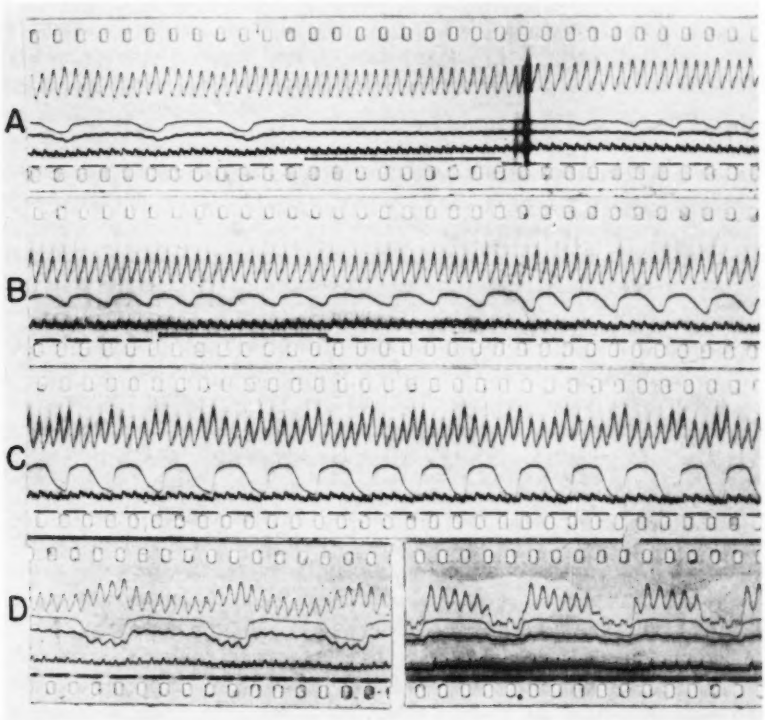

Fig. 2. Each record shows, from above down, ventricular volume, respiration (intrapleural pressure), arterial pressure, and time in seconds. In A and D venous pressure is recorded below respiration. In A, B and C the ventricles were under intrathoracic pressure throughout.

A. Normal breathing, followed by respiratory standstill from weak central stimulation of the right vagus (signal). This in turn is followed by rapid and shallow breathing.

B. Respiration initially rapid and relatively shallow. At the signal, the trachea was partially obstructed by a clamp.

C. Begins 15 seconds after the end of B, the tracheal clamp being left in place. Note effects of deeper inspiration on diastolic and stroke volume.

D. Both vagi sectioned. Ventricles under intrathoracic pressure (first part) and under atmospheric pressure (second part). Note reversal of inspiratory effects on diastolic and stroke volume.

when the breathing is shallow, more marked in ordinary quiet breathing (fig. 2A). They appear in exaggerated degree when inspiration is deepened

and prolonged, either by obstruction of the trachea (fig. 2, B and C) or by section of both vagi (fig. 2D, part 1).

Diastolic volume is maximum at the end of inspiration. With expiration it falls, but may require three or four beats to reach a steady minimum level. Stroke output falls simultaneously, and remains constant during the latter part of a prolonged expiratory pause (fig. 2D, part 1). At this time stroke output is near its minimum, but it becomes still smaller if the onset of the next inspiration happens to coincide with the beginning of systole. The sudden lowering of intrathoracic pressure appears to impede somewhat the emptying of the ventricles. This factor, if it continues to operate throughout inspiration, is more than offset in the later stages by some opposing influence, presumably the increase of diastolic volume. Stroke output reaches its maximum either at the end of inspiration or with the first systole following the onset of expiration. The largest systolic excursions are registered when expiration and systole begin simultaneously. Such a systole starts from maximum diastolic volume, and leaves a residual volume smaller than was left at the end of the preceding systole. The emptying of the ventricles thus appears to be aided by a coincident rise of intrathoracic pressure. Several examples illustrating the behavior described may be found in figure 2. In some animals inspiration augments stroke output nearly as much as diastolic volume (fig. 2, B and C); in others, the increase of stroke output is smaller in proportion, and the volume left at the end of systole relatively large (fig. 2D, part 1). These individual differences may depend on the condition of the heart, or on the effective venous pressure.

The respiratory effects described above appear only when the ventricles are under intrathoracic pressure. If the upper chamber of the tambour is opened to outside air, and the ventricles thereby put under atmospheric pressure, their volume and output diminish with inspiration. This confirms the observations of Cahoon, Johnson and Michael (1940), made under similar conditions. Eyster and Hicks (1933) also noted an inspiratory reduction of stroke output, but a simultaneous increase of diastolic volume. We are unable to account for the discrepancy between the latter finding and our own.

The reversal of the inspiratory effect, when the ventricles are placed under atmospheric pressure, seems due entirely to the abnormally high and unchanging resistance against which they must fill. Since the other thoracic vessels are left under subatmospheric pressure, there is a backward gradient of external pressures, tending to dam blood back into the auricles and veins. This back pressure obviously would increase during inspiration. Under these conditions right auricular pressure, measured against atmospheric, remains nearly constant throughout the respiratory cycle (fig. 2D, part 2). Since external pressure on the auricles and veins is

reduced during inspiration, it is difficult to see how their internal pressure could be held constant, except through distention by an increased volume of blood. Normally, right auricular pressure is well known to fall during inspiration (fig. 2D, part 1). It may also be noted that the respiratory variations of stroke output are much greater under atmospheric than under intrathoracic pressure. In the former condition, the inspiratory reduction of output is accompanied by a marked weakening of the carotid pulse, which may be almost completely suppressed by a deep inspiration (fig. 2D, part 2). Normally, the carotid pulse shows relatively small variations in the respiratory cycle.

Discussion. Since the cardiometer record shows only the combined volume of the two ventricles, other data must be used to distinguish volume changes on the right from those on the left side. From the differential pressure studies of Wiggers (1914) and of Hamilton, Woodbury and Vogt (1939), one would expect inspiration, particularly if forced, to augment filling and output of the right ventricle. Its effect on the left is much less clear. We have invariably found, with deep and prolonged inspiration (vagi sectioned) a fall of pressure in the carotid artery. The fall is greatest when the ventricles are under atmospheric pressure, and under those conditions evidently is due, in part or entirely, to a reduction of output from the left ventricle. When the ventricles are under intrathoracic pressure the inspiratory fall of carotid pressure is less profound but is still present. It might be attributed either to *a*, reduced left ventricular output, masked on the cardiometer record by a simultaneous and greater increase of output on the right side; or to *b*, inspiratory lowering of external pressure on the arteries in the thorax, reducing the total resistance against which the left ventricle empties. The latter explanation seems to imply an increased capacity of the thoracic arteries, and an inspiratory reduction of flow through arteries outside. It is difficult to reconcile either *a* or *b* with the observation of Machella (1936), that blood flow through the femoral artery is augmented during inspiration.

The present paper has dealt only with the variations of ventricular filling and output in the individual respiratory cycle, and not with the influence of respiratory movements on the mean cardiac output over longer periods of time. We realize that in the conscious human subject, under varying conditions of posture and muscular activity, the circulatory effects of inspiration and of expiration might differ somewhat from those observed on the anesthetized dog in the supine position.

SUMMARY

1. A method of cardiometric recording, designed for use with the chest closed, is described. It maintains on the ventricles an external pressure which is always approximately equal to intrathoracic pressure, varying

in the normal manner with the phases of respiration. It can be alternatively used in such a way that the ventricles are left under constant atmospheric pressure.

2. With the ventricles under atmospheric pressure, diastolic volume and stroke output diminish markedly with inspiration (confirming earlier investigators). These are abnormal effects, due to the artificially high resistance against which the ventricles are filled.

3. With the ventricles under intrathoracic pressure, combined diastolic volume and stroke output of the two ventricles are augmented with inspiration. These effects are most pronounced when inspiration is deep and prolonged. They are relatively small in quiet eupneic breathing, and are minimal when the breathing is rapid and shallow.

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THE EXTENT TO WHICH RADIOACTIVE CHLORIDE PENETRATES TISSUES, AND ITS SIGNIFICANCE

JEANNE F. MANERY AND LORRAINE F. HAEGE

From the Department of Physiology, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

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In a preceding publication Manery and Bale (6) reported the results of a survey of the extent and rate of penetration of injected radioactive sodium (Na^{24}) into mammalian tissues. There was some variation in the rate at which Na^{24} penetrated the tissues from the plasma, but in every case penetration proceeded until the tissue:plasma ratio of the radioactive isotope was the same as that for the normal sodium isotope. This value was never exceeded and therefore penetration was considered complete when equality of the ratios was attained. In many tissues entrance was complete in about 8 minutes and this finding, with some reservations (see (6)), was taken to indicate that all of the sodium contained in such tissues was extracellular. Na^{24} entered other organs more slowly, complete penetration requiring several hours. In some instances the presence of a small amount of intracellular sodium was suggested, but in other cases the slow rate of entrance could be attributed to other causes.

Since sodium and chloride differ so markedly in concentration in many tissues it seemed desirable to make a similar survey of the extent and rate of penetration of the radioactive isotope of chlorine (Cl^{38}). This was administered as the chloride of lithium. The isotope used disintegrates so rapidly that the information which can be obtained is limited. However, although the variation is fairly large, the data seem to show that radioactive chloride likewise becomes distributed from the plasma into most tissues until its tissue:plasma ratio is the same as that for normal chloride chemically determined. There is more evidence of intracellular chloride in these data than there was of intracellular sodium in the data referred to above. A comparison of the behaviour of the two unstable isotopes allows a more reasonable measurement of the extracellular water of many tissues than has hitherto been possible. An abstract of the experiments has already been published (5). A more detailed account will be presented here.

METHODS AND CALCULATIONS. Radioactive chloride ($_{17}Cl^{38}$)[1] of half

[1] The radioactive salt was kindly prepared for us by Dr. S. N. Van Voorhis and Dr. C. W. Strain of the Department of Physics, operating under a grant from the Rockefeller Foundation. We are particularly indebted to them for their excellent coöperation during these experiments.

life 37 minutes (8) was administered in a 1 per cent solution of lithium chloride. Five rats were injected intraperitoneally with 2.1 ml. per 100 grams of body weight and 5 rabbits intravenously via an ear vein with 2.8 ml. per kgm. of body weight. Although the half life of this chloride isotope is short, the activity was sufficiently high (5 millicuries per liter) to permit experiments of 5 to 6 hours' duration. During this period the salt was dissolved, the solution injected into the animal and time allowed for its distribution. The animal was then decapitated and bled. The tissues were dissected, ashed, subjected to the chemical procedures described below, and the radioactivity of the final solution determined with a Geiger-Müller counter. In some experiments two counters were used. The methods of dissecting the tissues, of determining chloride chemically, and of counting the solutions, were essentially the same as those described by Manery and Bale (6). Separate samples were taken for the determination of radioactivity and of chloride by the chemical method.

The lithium component of the salt also becomes radioactive during deuteron bombardment, but since the half-life of the lithium isotope is only 0.88 second its activity is soon dissipated. Although a reagent grade of lithium chloride was bombarded for these experiments it was found to contain a trace of an element other than chlorine or lithium which became radioactive. Sodium seemed to be the most likely contaminant. Hence the chloride of the solution was precipitated as silver chloride in the presence of an excess of sodium. The precipitate was dissolved and the final solution showed a true decay curve of an isotope the half life of which was 37 minutes. This solution was used as a standard of comparison for the unknown solutions.

Similarly, the chloride of each sample of tissue or body fluid was precipitated as the silver salt and the radioactivity of a solution of the precipitate determined. The samples were placed in 50 ml. centrifuge tubes containing sodium nitrate which was used to decrease the possibility of radioactive sodium being carried down in the silver chloride precipitate. Silver nitrate and nitric acid were then added as in most chloride methods in common use. The tubes were heated on a steam bath until the supernatant solution was clear, which required 1 to 2 hours. They were subsequently centrifuged and cooled on ice to solidify the fat. The supernatant liquid was removed by suction, care being taken not to remove any of the fat globules. After washing the precipitate twice with distilled water it was dissolved in 2 to 20 ml. of 1 per cent solution of potassium cyanide. The fat present was likewise dissolved. Vigorous stirring and slight warming were sometimes necessary to dissolve the larger amounts of precipitate. This solution was counted directly or diluted as desired. Although a solution of potassium cyanide was employed in all experiments reported here, ammonium hydroxide seems to be equally useful for dissolving the precipitate.

The half-life of Cl^{38} is so short that its disintegration is perceptible during the 4 minute periods of counting. Hence each tissue was counted for 2 to 5 periods carefully timed and the results for each period calculated from the theoretical decay curve. The averages of all the periods for each tissue are the figures presented in the tables. All values except those asterisked were calculated from counts which were many times the background count. Those which were twice the background or less were discarded. The greatest percentage differences between members in a series of 16 duplicate, triplicate or quadruplicate samples were 27 and 29 per cent while the average including these values was 11 per cent and excluding them was 8.5 per cent.

The methods of calculation and presentation of the data have been explained in the previous paper (6) where the precise meanings of the symbols used are given. It will suffice to state that a value called $(H_2O)_E$ or the extracellular water was calculated as follows in grams per 100 grams of fresh tissue:

$$(H_2O)_E Cl^{38} = \frac{\text{tissue counts per min. per kgm.}}{\text{plasma counts per min. per liter}} \times 0.95 \times 0.93 \times 100$$

These values were compared to similar values calculated from the chemically determined chloride.

Results. It should be pointed out at the outset that no toxic effects of lithium chloride were observable in these acute experiments. Even after intravenous injection into rabbits there were no outward manifestations of abnormal behaviour in 52 minutes. A comparison of the chloride concentrations of tissues reported here with those in 2 former papers (6, 7) shows that the electrolyte pattern was not appreciably altered by the treatment.

Essentially only two periods of time were studied, a short period of a few minutes which was shown (6) to be adequate for radioactive sodium to enter the extracellular phase of tissues, and a longer period ranging from one-half to one hour which is sufficient for equilibrium to be established between plasma and most tissues. Penetration was taken to be complete when the value of $(H_2O)_E$ calculated from Cl^{38} became equal to that calculated from Cl. The results for 5 rats are shown in table 1 and for 5 rabbits in table 2. To conserve time the tissues were not always analyzed chemically for chloride. By consulting the data in references 6 and 7 it can be seen that the variation in tissue chloride between stock animals prepared in this manner is small.

In many of the tissues listed in both tables the Cl^{38} values closely approximate the Cl values. As in the radioactive sodium experiments the tissues can be divided into two groups, those in which the Cl and Cl^{38} values are equal indicating that the penetration of radioactive chloride into

the Cl-containing phase is completed rapidly, and those in which the Cl value exceeds the Cl^{38} value in all of the periods of time studied. Since one hour was the longest period allowed before decapitation, we have no data to show that the Cl^{38} slowly penetrated the entire Cl-containing phase of tissues into which it was delayed, as was the case with radioactive sodium.

TABLE 1

Data obtained from rats injected intraperitoneally with lithium chloride containing radioactive chloride

TISSUE	RAT 1		RAT 2	RAT 3		RAT 4	RAT 5
	Rat weight (grams):						
	294		264	334		280	284
	8 min.		8 min.	28 min.		30 min.	32 min.
	Values of $(H_2O)_E$ (grams per 100 grams fresh tissue) calculated from						
	Cl	Cl^{38}	Cl^{38}	Cl	Cl^{38}	Cl^{38}	Cl^{38}
Skin	38.3	36		48.6		38	32*
Kidney	48.9	126 (?)		48.6	74 (?)	41	38*
Liver	26.4	97 (?)	52 (?)	27.7	37 (?)	24*	
Testes	54.2	80 (?)	27	53.8	27	22	16*
Gastrocnemius	12.4	13	7.8	14.8	13		12*
Heart	23.4		21		22		22*
Brain	27.4	4.2*	2.6	29.3		6.0	negl.
Plasma Cl (m.eq. per l.)	102.8			103.9			
Per cent distribution			32	29		28*	25
$\frac{Cl^{38} \text{ perit. fl.}}{Cl^{38} (H_2O)_E}$			8.1	4.1		4.6*	3.9
Per cent dose in plasma	3.3		3.1	7.0		7.2	8.2

Negl. = negligible.

* Values asterisked were calculated from counts which were only 2 to 4 times the background count.

(?) No significance is attached to the fact that these figures exceed the Cl values, since they are not substantiated by the data on rabbit tissues. It seems likely that contamination with peritoneal fluid occurred in spite of efforts to avoid it. A pool of peritoneal fluid was always evident and its radioactivity, as the table shows, is 4 to 8 times that of plasma.

Generally speaking, the first group contains skin, kidney, liver, gastrocnemius, heart, abdominal muscle and tendon. The tissues of the second group, which show a great difference between the Cl and Cl^{38} values, are testes, pyloric mucosa and brain.

In considering the tissues of the first group it can be seen that, with the exception of figures followed by a question mark in table 1, the Cl and Cl^{38} values are similar except for the kidney of rabbit 4. The skin of rab-

bits (table 2) possesses more chloride than that of rats but its entire Cl phase seems to be quickly penetrated. Although the Cl^{38} and Cl values approximate each other in kidney cortex it is likely that the tubules contain intracellular chloride. Reference has been made to this point with regard to the rapid entrance of Na^{24} into the Na phase of kidney (6).

TABLE 2

Data obtained from rabbits injected intravenously with lithium chloride containing radioactive chloride

TISSUE	RABBIT 1		RABBIT 2		RABBIT 3		RABBIT 4		RABBIT 5
	Rabbit weight (kgm.):								
	2.6		2.9		3.0		2.9		2.7
	11 min.		41 min.		45 min.		48 min.		52 min.
	Values of $(H_2O)_E$ (grams per 100 grams fresh tissue) calculated from								
	Cl	Cl^{38}	Cl	Cl^{38}	Cl	Cl^{38}	Cl	Cl^{38}	Cl^{38}
Skin	60.6	56				53	62.6		61
Kidney	38.8	41					45.4	59	41*
Liver	19.3	18	19.8	16		17*	21.4	25*	16
Testes		28				24	45.4		46*
Gastrocnemius	11.6	8.3*	11.5	8.4	12.3		11.4	9.4*	9.0
Heart	29.6	31	30.2	29	28.8				28*
Abdominal muscle	15.8	14*	16.9	14	15.9				18
Pyloric muscle	40.4	30	44.6	40	38.3	26*	43.8		39
Pyloric mucosa		33			52.4	34*	58.9	38	30
Ear cartilage	50.2	37*					48.5	58*	62
Tendon	57.1	56			58.4			66	59
Brain	31.8	4.7*				10	33.0	11*	9.5
Plasma Cl (m.eq. per l.)	105.2		98.8		105.1		101.5		
Per cent distribution	20		17		26		17		23
Per cent dose in plasma	13		12		9		13		10

* Values asterisked were calculated from counts which were only 2 to 4 times the background count.

In the heart, abdominal, gastrocnemius and pyloric muscles the $(H_2O)_E$ values calculated from Cl^{38} simulate those calculated from Cl. However, because there was some suggestion in the Na^{24} data (see table 2 of reference 6) that Na^{24} did not enter the entire Na phase of muscle in 20 minutes, it should be pointed out that in ten corresponding pairs the Cl^{38} value is lower than the Cl value by an average of 18 per cent. In view of the large percentage difference between duplicate analyses (7 and 11 per cent for Na^{24} and Cl^{38} respectively) these two sets of data scarcely support, but

certainly do not disprove, the proposal recently made for frog muscle by Conway et al. (3) that a small part of the total sodium and chloride of muscle might be intracellular.

The data on cartilage and tendon as they stand (table 2) indicate that the entire Cl phase is freely accessible to Cl^{38}. It should be recalled that tendon is dense connective tissue with few cells and that cartilage is outstanding because the sodium concentration is greatly in excess of the chloride concentration. Cl^{38} is not appreciably delayed in its entrance into the Cl phase of cartilage and it seems reasonable to consider that it represents the extracellular phase of the tissue. The extracellular water of rabbit ear cartilage when calculated from chloride is only slightly less than the total water of the tissue as found by Manery and Hastings (7). This leaves half the sodium content in a solid non-aqueous phase.

Of greater interest perhaps are tissues of the second group. Relatively small amounts of radioactive chloride are found in brain tissue even in one hour after injection. Judging from previous estimations of the hemoglobin content of brain (7) this is probably somewhat more than would be contained in the blood of the tissue but still is so small that only one third of the total chloride phase has been penetrated. Other instances of the slow penetration of substances into brain have been cited (6) and will not be repeated here. As will appear in the discussion these data cannot yet be taken to indicate intracellular chloride.

Testes and pyloric mucosa are exceptional because the chloride content is greatly in excess of the sodium, making the Cl phase much larger than the Na phase. In testes only one-half of the Cl phase is penetrated by Cl^{38} in 45 minutes. The differences are large and consistent except for rabbit 5. The average value of $(H_2O)_E$ for both types of animals calculated from Cl^{38} is about 28 while that calculated from Cl is nearly 50. The Na value, on the other hand, is 30 (6, 7) for both rats and rabbits, which is almost the same as the Cl^{38} value. Radioactive chloride, then, has penetrated a volume equal in magnitude to the Na phase in the periods of time studied. In our previous report data on Na^{24} indicated that all but 20 per cent of the entire Na phase was entered rapidly by Na^{24}. It is possible that only this portion is penetrated by Cl^{38}.

In pyloric mucosa a similar situation exists. The value of $(H_2O)_E$ calculated from Cl^{38} amounts to slightly more than half that calculated from Cl and is almost equal to the Na value. In this tissue, too, the figures indicate that Cl^{38} has entered a volume equal to the Na phase and has not progressed beyond it in 52 minutes. Whether the "excess" chloride (i.e., chloride in excess of sodium) found by analyses in stomach mucosa occurs within the secreting cells or in the lumen of the glands rather than in supporting cells is not yet known, but in any case plasma chloride does

not seem to freely diffuse into this volume or to exchange with the chloride already there.

It seems improbable that the similarity of the Cl^{38} values to the Na values in these tissues is mere coincidence. A simple interpretation of the finding is to consider that all of the sodium found by analysis is contained in the extracellular phase. Into this phase Cl^{38} promptly enters, but it cannot exchange so rapidly with the remaining chloride, of the pyloric mucosa for example, because this portion is either intracellular or in the gland lumens. The rate of entrance into the second phase might depend on secretory processes which in this case were slower than diffusion. A similar interpretation might apply to testes. If this interpretation can be accepted the extracellular water of both testes and pyloric mucosa would measure about 30 per cent of the wet weight. A more complete study of the rate of entrance into the entire Cl space would be desirable in order to demonstrate the existence of two phases entered at different rates.

By way of summary a graphic comparison of representatives of the two groups of tissues discussed above has been made in figure 1.

The water of the body into which the absorbed Cl^{38} became distributed can be calculated by assuming the same concentration in the water as in a plasma ultrafiltrate. This was called the per cent distribution (see equation 5 (6) for the exact calculation) and may be taken as a good approximation to the true volume of extracellular water. It was found to be about 29 per cent of the body weight in rats (table 1) and about 21 per cent in rabbits (table 2). The results are more variable but are in fairly close agreement with similar values calculated for radioactive sodium. The urine was collected in each of the rabbit experiments and analyzed in order to ascertain the amount of Cl^{38} excreted. It was found to contain so few counts that collection and analyses of urine were omitted in the rat experiments.

The ratio of Cl in peritoneal fluid:Cl^{38} in $(H_2O)_E$ for rats (table 1) indicates that more time was required for equilibrium to be established in the case of lithium chloride than was observed when sodium chloride was injected (table 2 of (6)).

The rate of disappearance of radioactive chloride from the plasma of rabbits was likewise studied. Blood was collected from veins of the ear on the opposite side to that which received the injection, and was centrifuged at once. In the 3 cases where collections were made in 5 to 8 minutes after injection only about 15 per cent of the dose injected remained in the plasma. In the subsequent 40 minutes this dropped only a few per cent. The values obtained at decapitation are given in the tables. Again the isotopes afford an illustration of the rapidity with which diffusion occurs from the plasma into the extracellular fluid of the body. It should be

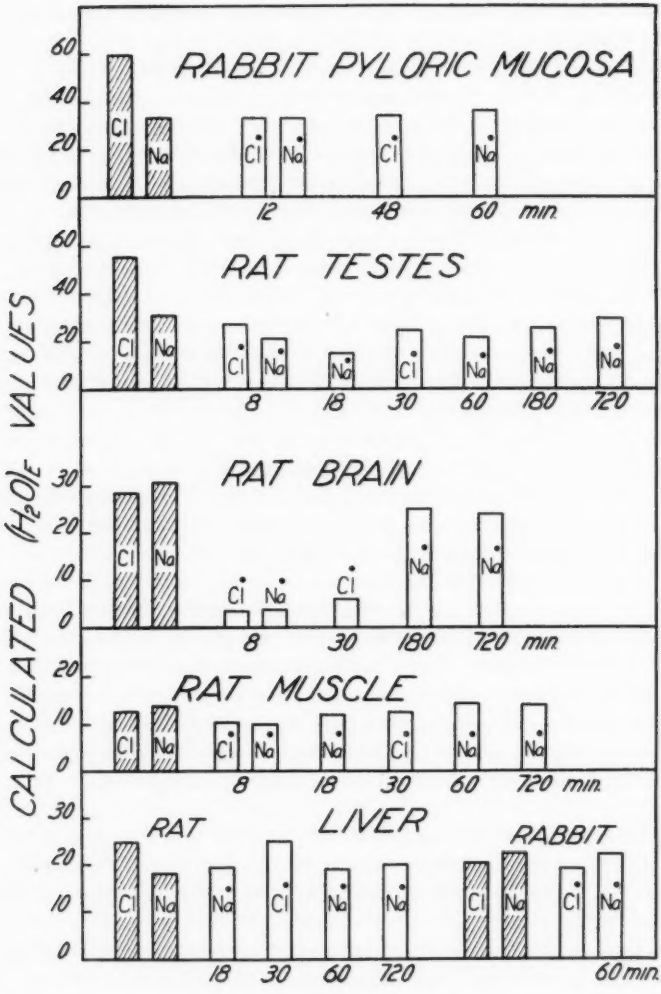

Fig. 1. A comparison of the values of $(H_2O)\hat{E}$ calculated from radioactive chloride ($Cl^{\bullet}$) with those calculated from radioactive sodium ($Na^{\bullet}$).

Values of $(H_2O)\hat{E}$ are calculated from Na and Cl chemically determined (cross hatched blocks), and from the radioactivity of the isotopes Na^{24} and Cl^{38}, according to the equation given in "methods and calculations." These values in grams per 100 grams of fresh tissue for sodium probably closely approximate the extracellular water of each tissue. The figures on the horizontal axis refer to the period of time which elapsed between injection of the radioactive solution and decapitation. The radioactive Na values ($Na^{\bullet}$) were taken from Manery and Bale (6).

recalled (see equation 6 of reference (6)) that the actual plasma volume was not measured for these calculations. The blood volume was assumed to be constant. Hematocrit values were determined in the rabbit experiments but an average value was assumed in the case of rats.

DISCUSSION. It was shown by Manery and Hastings (7) that mammalian soft tissues could be grouped into *a*, those in which the Na:Cl ratios were the same as in an ultrafiltrate of plasma, and *b*, those which contained chloride in excess of sodium. In other words the values of $(H_2O)_ECl$ equal those of $(H_2O)_ENa$ in group *a* but $(H_2O)_ECl$ is always greater than $(H_2O)_ENa$ in group *b*. To a group *c* we might relegate skeletal structures in which $(H_2O)_ENa$ greatly exceeds $(H_2O)_ECl$. A further characterization of these groups is now possible. Radioactive isotopes of sodium and chloride penetrate all of the tissues of group *a* (except brain) with great rapidity, becoming distributed between the tissue and the plasma to the same magnitude as the normal isotope. Neglecting minor differences the tissues thus characterized are skin, kidney, liver, muscles and tendon. In group *b*, where the Cl phase exceeds the Na phase in magnitude radioactive sodium enters the entire Na phase fairly rapidly but even in one hour radioactive chloride has penetrated only the Na phase and is definitely delayed in entering the entire Cl phase. Testes and pyloric mucosa are the outstanding members of this group. Our data on the rates of entrance of the radioactive isotopes into tissues of group *c* are rather incomplete but, since these are highly specialized, they require a rather different type of laboratory handling and of theoretical consideration.

The data obtained using radioactive sodium and chloride, if interpreted as indicated above, lend some support to the hypothesis of Manery and Hastings that there are three chemical phases in tissues: 1, the extracellular phase, $(E)_p$, which is in ionic (Donnan) equilibrium with plasma; 2, an intracellular phase, $(C)_1$, which contains neither Na nor Cl; and 3, an intracellular phase, $(C)_2$, which contains Cl but not its equivalent of Na. Furthermore, a method using these two isotopes is suggested for measuring the extracellular water of tissues where the method based on the analyses of the total Na and Cl failed, and for fractionating the Na and Cl of a tissue so that a measure of the intracellular portion of sodium and chloride can be attained.

It should be reiterated that these properties of tissues have been stated in the most general terms and that each tissue requires particular consideration. Brain, for example, contains sodium and chloride in ultrafiltrate proportions which suggests that the Na and Cl exist in the extracellular phase of the tissue. However neither radioactive sodium nor chloride enter the NaCl-containing phase of brain for long periods of time after administration, and in this regard they do not differ from phosphate which is usually intracelllular. Before the slow penetration can be taken to

indicate intracellular NaCl other factors which might prevent free diffusion into the extracellular phase of the tissue must be considered. It is difficult to ascribe this difference in the rate of penetration between brain and other tissues of group *a* to the number and distribution of capillaries, although the white matter is considered by Cobb and Talbott (2) to contain fewer capillaries than grey matter or resting muscle. It is this portion of the blood-brain barrier, namely, that located in the region of the cerebral capillaries which concerns us, and not the barrier at the chorioid plexuses. Wallace and Brodie (9) have likewise been interested recently in this portion of the barrier.

Histological differences between cerebral capillaries and those elsewhere, which could give rise to different permeability properties, have not been clearly demonstrated. It has been known however that certain dyes, for example trypan blue (see (4) for review) will not pass through cerebral capillary walls although they easily traverse those of other tissues. It would be surprising if ions like sodium, chloride and phosphate were delayed at the cerebral capillary walls from entering brain extracellular space. Real differences seem to occur between brain and other tissues in the relation of the connective tissue elements to the capillary walls and to nerve cells (Cobb, 1). Perhaps diffusion through the extracellular space is slowed by virtue of this particular arrangement. Until more information is available, therefore, we do not know whether the mode of penetration of radioactive sodium and chloride into brain is indicative of intracellular sodium and chloride or is due to some other factor.

SUMMARY

The extent and rate of penetration of Cl^{38} into rat and rabbit tissues were measured by comparing the ratio of the tissue:plasma concentration of Cl^{38} to a similar ratio for Cl chemically determined. When the two ratios were equal penetration was complete.

Penetration was completed in a few minutes after injection in kidney, liver, muscles, cartilage and tendon. Cl^{38} did not enter the entire Cl-containing phase of testes and pyloric mucosa even in one hour but penetrated a volume of tissue equal in magnitude to the Na phase. Only a trace of the radioactive isotope was found in brain.

The significance of these findings is discussed.

We wish to express our indebtedness to Dr. W. F. Bale of the Department of Radiology for supervising the operation of our Geiger-Müller counter, and for assisting with a second counter in some of the experiments.

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"ALKALINE" PHOSPHATASE ACTIVITY OF THE PROXIMAL CONVOLUTED TUBULES AND THE MECHANISM OF PHLORIZIN GLYCURESIS

ROBERT A. KRITZLER AND ALEXANDER B. GUTMAN

From the Departments of Pathology and Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City

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It is generally accepted that the principal action of phlorizin is upon the kidneys (1, 2), where reabsorption of glucose from the glomerular filtrate by the cells of the proximal convoluted tubules is inhibited (3, 4). The crux of the problem of phlorizin glycuresis now appears to lie in determining what specific cellular mechanisms are involved in the "active" processes of tubular reabsorption and what phase of these processes is interrupted by phlorizin.

In 1933 Lundsgaard suggested (5) that "the 'active' step in tubular reabsorption of glucose consists of phosphorylation [which he associated with the large amounts of phosphatase present in kidney tissue] and that the effect of phlorizin is to block phosphorylation." In support of this view, Lundsgaard reported (5) that phlorizin in M/200 to M/50 concentration definitely inhibits both dephosphorylation of glycerophosphate by renal extracts and the formation of phosphoric esters in muscle pulp.

The attempt to relate phosphorylation of glucose to the synthesizing action of renal phosphatase soon led to difficulties and many questioned the validity of this whole concept of glucose reabsorption and phlorizin glycuresis: 1. Lundsgaard himself subsequently found (6) that the dosage of phlorizin necessary for maximal glycuresis in perfused pump-lung-kidney preparations was only about $\frac{1}{5}$ that required for inhibition of esterification in muscle pulp. This discrepancy later appeared to be explained by the observation (7) that phlorizin following injection is concentrated in the proximal convoluted tubules where glucose reabsorption takes place; but whether it is sufficiently concentrated to inhibit renal phosphatase was not determined. 2. Lambrechts found (7) no parallelism between the glycuretic action and the in vitro inhibiting effect on renal phosphatase of various substances related to phlorizin. Phloroglucin and salicin, which are not glycuretics, slightly inhibited renal phosphatase activity in vitro whereas arbutin and phlorin caused glycosuria but did not inhibit the enzyme. 3. The in vitro inhibition of renal phosphatase activity by

phlorizin reported by Lundsgaard (5) was found to be inconsiderable by subsequent investigators (3), particularly in properly buffered solutions (8). 4. No consistent difference was observed between the phosphatase activities of renal extracts of phlorizinized and normal rats (3).

These discrepancies led Walker and Hudson (3) and others to infer that phlorizin does not cause glycuresis by inhibiting renal phosphatase, a conclusion which is supported by our results. We have employed the Gomori-Takamatsu histochemical technique (9, 10) for demonstrating phosphatase in tissues. This method permits of precise cellular localization of the enzyme and also gives a rough measure of the amount of enzyme present.

It can be shown in this way that the proximal convoluted tubules are extremely rich in "alkaline" phosphatase (9–12), which is largely concentrated at or near the luminal border of the cells. We found no indication either by this direct histochemical approach or by conventional chemical methods of any significant inhibition of the renal phosphatase activity of the proximal convoluted tubules in maximally phlorizinized animals as compared with normal controls. This result was obtained in both acutely and chronically poisoned rats and following direct injection of phlorizin into one renal artery of the dog by the Zuntz technique (1).

PROCEDURE. 1. *Acute phlorizin poisoning.* Sixteen albino rats weighing about 200 grams were injected intraperitoneally with 10 mgm. phlorizin (recrystallized) in 2 cc. of 2 per cent sodium bicarbonate. Fifteen minutes after injection a urine sample for sugar determination was obtained from the first rat, which was then sacrificed. This procedure was repeated at intervals up to 90 minutes after injection. A part of each kidney was fixed immediately for histochemical examination. Another portion was weighed, ground, extracted with water and the "alkaline" phosphatase activity of the extract determined by the King and Armstrong method (13). Unincubated aliquots of the aqueous extracts gave control readings. The results are expressed in units = number of milligrams phenol liberated per hour. Kidney sections from 4 untreated rats served as controls.

2. *Chronic phlorizin poisoning.* Three albino rats weighing about 200 grams were given daily subcutaneous injections of 20 mgm. phlorizin in 2 cc. peanut oil for 5 days. Quantitative sugar analyses were made on measured 24 hour urine samples. The kidneys were studied histologically and chemically by the methods indicated.

3. *Zuntz experiments.* After sodium pentobarbital anesthesia and preliminary saline infusion (300 cc.), the kidneys, renal arteries and ureters of three dogs were exposed by posterior approach. No. 6 French ureteral catheters were then tied in place. When approximately equal urinary flow from both kidneys was established, control urine samples were collected for sugar and non-protein nitrogen determinations and small biopsies

from each cortex were obtained. Hemorrhage was controlled by cautery. Phlorizin was then rapidly injected into the left renal artery in a dosage of 0.5 mgm./kgm. body weight (in 0.5–0.75 cc. 2 per cent sodium bicarbonate). The right artery was temporarily occluded by extrinsic pressure during injection into the left. Specimens of urine and biopsies of the renal cortices were taken immediately after the injection and at intervals thereafter. Sugar and non-protein nitrogen were estimated in the urine samples by the Folin-Wu methods. Histochemical and chemical studies were made of the renal cortex biopsies as described.

The Gomori-Takamatsu technique[1] involves incubation of uniform, thin, mounted sections for 2 hours at 38°C. in a mixture of sodium β-glycerophosphate and calcium nitrate buffered at pH 9.1 with sodium barbital. At the sites where phosphate is liberated from the substrate through the action of the enzyme, a colorless calcium phosphate complex is formed. The sections are then immersed into silver nitrate solution and simultaneously exposed to ultraviolet light. Brown granules appear where the complex has formed as a result of the enzyme activity. Serial sections of phlorizinized kidneys were made to exclude local variations; and to facilitate comparison with controls, the sections were mounted on the same slide. The enzymic activity of the cells of the proximal convoluted tubules could be estimated adequately by the following criteria: intensity of color and size of the granules, the distance between them, and the proportion of the cytoplasm occupied. The activity was graded from 4+ to 1+. By rigid uniformity in the steps of the technique, satisfactorily reproducible results could be obtained.

Results. 1. *Acute phlorizin poisoning* (table 1, fig. 1A, B). Fifteen minutes after injection, when glycuresis was already maximal, the phosphatase activity of the proximal convoluted tubules by the histochemical method was unchanged as compared with the controls. Chemical analyses of the renal cortex however showed a moderate, but consistent and perhaps significant reduction. Thirty minutes after injection, when glycuresis was still maximal, the results by both methods showed no significant decrease. One kidney in 2 animals gave slightly less enzymic activity by the histochemical method. Sixty and 90 minutes after injection, when glycosuria was still maximal, enzymatic activity was normal by both methods. No convincing inhibition of the "alkaline" phosphatase activity of the proximal convoluted tubules was observed in the acute experiments.

2. *Chronic phlorizin poisoning* (table 2). In 2 of the animals in which urinary excretion of sugar was pronounced, averaging 2 grams a day, the chemical and histochemical determinations showed essentially normal

[1] We employed the improved procedure of Kabat and Furth (12) to whom we are greatly indebted for details in advance of publication. Magnesium salts were not added to the substrate mixture.

"alkaline" phosphatase activity. One kidney showed a slight decrease by the histochemical method, of doubtful significance. In the third animal, in which glycuresis was less marked, similar results were obtained by both methods.

TABLE 1

Renal "alkaline" phosphatase activity of rats after acute phlorizin poisoning (intraperitoneal injection of 10 mgm. phlorizin)

RAT NUMBER	MINUTES AFTER INJECTION	URINE QUALITATIVE SUGAR	"ALKALINE" PHOSPHATASE ACTIVITY OF RENAL CORTEX TISSUE			RAT NUMBER	MINUTES AFTER INJECTION	URINE QUALITATIVE SUGAR	"ALKALINE" PHOSPHATASE ACTIVITY OF RENAL CORTEX TISSUE		
			Histochemical		Chemical				Histochemical		Chemical
			Right kidney	Left kidney					Right kidney	Left kidney	
					units /gram						*units /gram*
1		0	4+	3+		11		4+	4+	4+	
2	Control	0	4+	3+		12		4+	4+	4+	384
3		0	4+	4+	370						
4		0	4+			13		4+	4+	4+	
						14	60	4+	4+	4+	
5		4+	4+	4+	202	15		2+	4+	4+	
6	15	4+	4+	4+	288	16		4+	4+	3+	362
7		4+	4+	4+	312						
8		4+	4+	4+	266	17		4+	3+	4+	
						18	90	3+	3+	4+	
9		3+	2+	3+		19		3+	4+	4+	
10	30	4+	2+	4+		20		4+	4+	4+	422

TABLE 2

Renal "alkaline" phosphatase activity of rats after chronic phlorizin poisoning (five daily subcutaneous injections of 20 mgm. phlorizin)

RAT NUMBER	AVERAGE DAILY URINE SUGAR	PHOSPHATASE ACTIVITY OF RENAL TISSUE		
		Histochemical		Chemical*
		Right kidney	Left kidney	
	grams			*units/gram*
21	1.9	4+	4+	224
22	2.2	2+	4+	260
23	0.7	4+	4+	326

* Determinations made on cortex and medulla of half a kidney. Average value of 3 normal rat kidneys prepared the same way: 276 units.

3. *Zuntz experiments* (table 3, fig. 1C, D). It was hoped that by injecting the drug into one renal artery the enzyme activity could be studied in kidneys that were excreting different amounts of glucose. We were

unable to take biopsies of the right (non-injected) kidney before sugar appeared in the urine samples of that side. A probably significant differential in sugar excretion was obtained, however, in urine specimens taken 3 minutes after the injection. No convincing decrease in the phosphatase activity of the proximal convoluted tubules was found in the kidneys excreting large amounts of glucose as compared with the same before the injection of phlorizin.

TABLE 3

Representative protocol showing results of injection of phlorizin into the left renal artery of the dog (Zuntz technique) upon the "alkaline" phosphatase activity of the renal cortex of both kidneys

KIDNEY	URINE			TISSUE BIOPSIES OF RENAL CORTEX		
	Minutes after injection collection was begun	Volume	Glucose	Minutes after injection biopsy taken	Phosphatase activity: Histochemical	Phosphatase activity: Chemical
		cc.	*mgm. per cent*			*units /gram*
Left	Before injection	2.8	95	Before injection	4+	135
Right	Before injection	2.8	61	Before injection	4+	159
Injection: 8.4 mgm. phlorizin (0.5 mgm./kgm.) into left renal artery						
Left	3	2.6 in 2 min.	980	6	3+	111
Right	3	2.9 in 2 min.	810	8	4+	164
Left	10	3.3 in 1½ min.	2090	15	3+	145
Right	10	3.3 in 1½ min.	1980	13	4+	70*
Left	20	3.8 in 1½ min.	1400	23	3+	63*
Right	20	3.0 in 1½ min.	1453	25	4+	197
Left	30	2.0 in 1½ min.	1760	35	4+	190
Right	30	2.1 in 1½ min.	1620	37	4+	106
Left	60	1.9 in 2 min.	1880	65	4+	
Right	60	1.5 in 2 min.	1880	65	4+	

* Biopsies taken too close to cauterized areas.

In the interpretation of the histochemical results several points had to be considered. 1. Phlorizin might have diffused out of the tissues during fixation by absolute alcohol or a hypothetical inactive phlorizin-phosphatase complex might have been dissociated during fixation and the enzyme reactivated. In fresh tissue fixed only by freezing, no difference could be found in the renal "alkaline" phosphatase activity of normal and phlorizinized rats. 2. During incubation, the water soluble phlorizin might have diffused out leaving a concentration in the tissues too low for

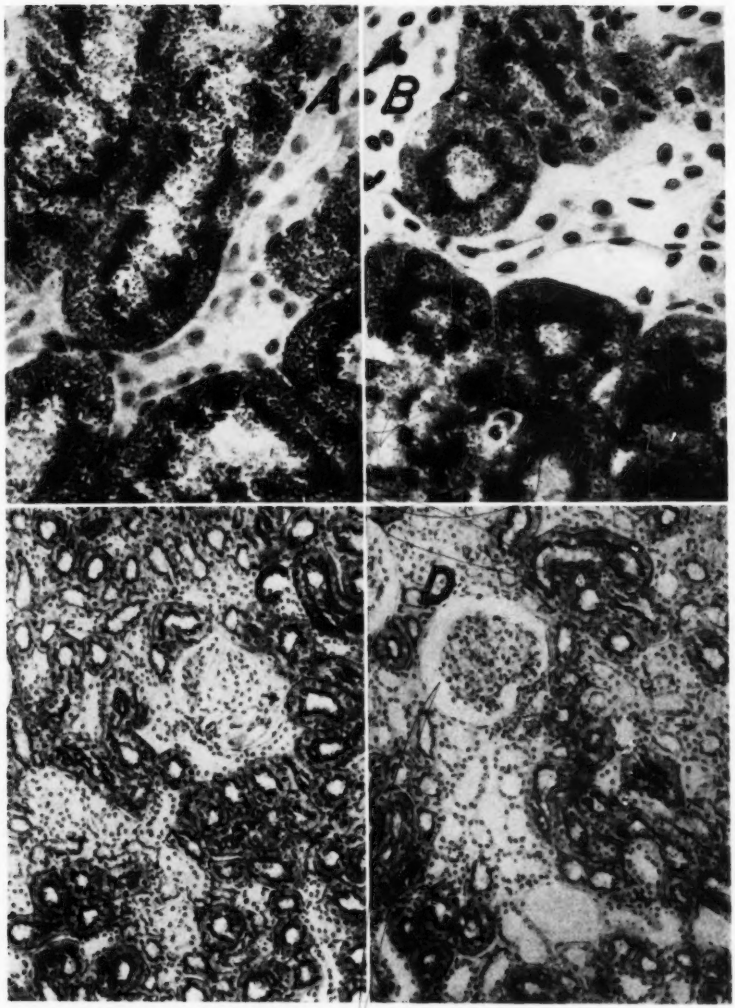

Fig. 1. Photomicrographs of kidney sections showing localization of "alkaline" phosphatase. A. Control rat, ×460; B. Acutely phlorizinized rat (marked glycuresis), ×460; C. Dog before injection of phlorizin, ×110; D. Same kidney 10 minutes after injection (marked glycuresis), ×110. Enzymic activity, indicated by granules in proximal convoluted tubules, is unaffected by phlorizin. Glomeruli, distal convoluted tubules and collecting ducts show no granules.

inhibition of the enzyme. When sections of normal rat kidneys were incubated in the presence of added phlorizin in concentrations up to M/100, no reduction in enzyme activity resulted.

Discussion. There is no evidence that phlorizin glycuresis is due to inhibition of the "alkaline" phosphatase present in the proximal convoluted tubules. There is considerable recent support, on the other hand, for Lundsgaard's main thesis (5, 6, 14) that phosphorylation of glucose occurs in the course of tubular reabsorption; not through reverse catalysis of a phosphatase, however (15, 16), but by the action of a specific enzyme system, kidney phosphorylase (15, 17, 18, 19). The evidence for existence of such an enzyme in the kidney and the present concept of its action was reviewed recently by Cori (20) and by Kalckar (21). It would appear that kidney phosphorylase constitutes part of the specific cellular mechanisms postulated to explain the rapid transport of glucose from the lumen of the proximal convoluted tubules to the blood-stream. In this scheme, the rôle of the "alkaline" phosphatase of the proximal convoluted tubules is to dephosphorylate hexose-phosphoric esters. Our results suggest that it is not this latter step which is blocked by phlorizin. This is in accord with the work of Kalckar (15, 17) which indicates that phlorizin inhibits glucose reabsorption by blocking some phase of the action of kidney phosphorylase upon glucose.

SUMMARY

No significant inhibition of the "alkaline" phosphatase activity of the proximal convoluted tubules was found by histochemical or chemical methods in acutely and chronically phlorizinized rats and in dogs. Phlorizin glycuresis apparently is not due to blocking of dephosphorylation by "alkaline" phosphatase in the kidney tubules.

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THE EFFECTS OF SOME DRUGS ON THE CROSSED PHRENIC PHENOMENON

A. M. SELIGMAN[1] AND W. A. DAVIS

From the Department of Physiology in the Harvard Medical School

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Langendorff (1887) and Girard (1890) observed contractions of one-half of the diaphragm in rabbits and dogs after an ipsilateral semisection of the spinal cord above C_3 and severance of the contralateral phrenic nerve. Schiff (1894) and Porter (1895) showed that the crossing of the respiratory impulses occurred only at the level of the phrenic nuclei. Rosenblueth and Ortiz (1936) found that the interruption of afferent nerve impulses was not responsible for the phenomenon. The phenomenon occurred in the absence of asphyxia. Reversible transient crossed contractions were obtained by reversible blocks of the phrenic nerve with ether or direct currents. Crossing did not occur until all the motor fibers of the phrenic nerve were cut. Rosenblueth, Klopp and Simeone (1938) proved that the phenomenon was independent of the respiratory center by stimulating electrically the respiratory tract above C_3. It was their conclusion that crossed contractions occur directly as a result of blocking motor fibers, that the central changes are mediated by some process not requiring the conduction of nerve impulses, and that once the crossed path has been opened subsequent crossings are more readily obtained.

The present study was undertaken to determine the effect of some drugs on the crossed phrenic phenomenon.

METHOD. Rabbits and cats were employed. The experiments were performed under dial anesthesia (Ciba, 0.6 to 0.7 cc. per kgm., intraperitoneally). In all the animals the cord was semisected at C_2, producing a respiratory hemiplegia. The diaphragmatic contractions were recorded as described by Rosenblueth, Klopp and Simeone, by using Head's slips, which attach to the xyphoid cartilage. In the records obtained by this procedure, diaphragmatic contraction is denoted by upward excursions of the tracing. All drugs were given intravenously into the jugular or femoral veins. Atropine (1 mgm. per kgm.) was given before the injection of prostigmin or eserine. Acetylcholine solutions were prepared fresh from the crystalline hydrochloride. Stimulation of the vagus nerves was ac-

[1] George Cheyne Shattuck Memorial Fellow, and Jeffries Wyman Scholar of the Harvard Medical School.

complished with alternating shocks from a Harvard induction coil with 5 volts in the primary circuit, by means of shielded silver electrodes. Asphyxia was produced by attaching to the tracheal cannula a rubber balloon containing expired air.

RESULTS. A. *Prostigmin.* Of the 2 cats and 9 rabbits on which observations were made, following the injection of prostigmin, permanent crossing was obtained in 1 cat and in 1 rabbit, reversible crossing was

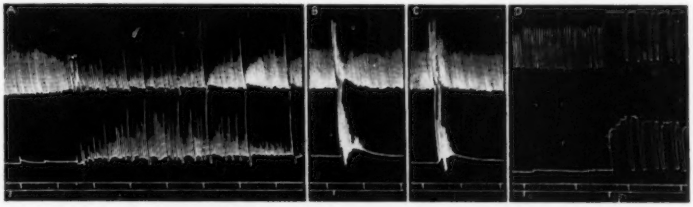

Fig. 1. Cat; dial; atropine (1 mgm. per kgm.). Left spinal semisection at C_2. Time signal: 30 sec. In this and the following figures the upper record corresponds to the right and the lower record to the left Head's diaphragmatic slip. The lower signals indicate the following procedures: A, prostigmin (0.5 mgm.); B, acetylcholine (100 γ)—injections of acetylcholine before prostigmin had not produced any crossed contractions; C, acetylcholine (100 γ) after denervation of the carotid sinuses; D, section of the vagi, first the left, then the right.

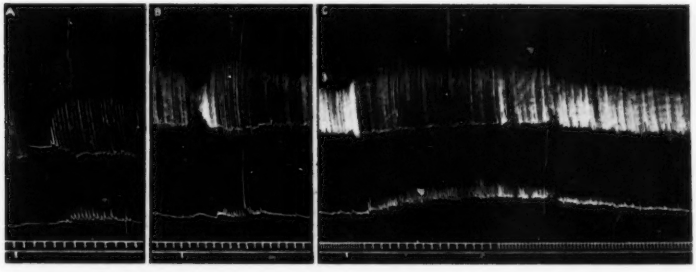

Fig. 2. Rabbit; dial; atropine (1 mgm. per kgm.); eserine (2 mgm. per kgm.). Left spinal semisection at C_2.

A, acetylcholine (200 γ); B, acetylcholine (300 γ) after denervation of the carotid sinuses; C, eserine (0.6 mgm. per kgm.).

noted in 1 cat (fig. 1A) and 1 rabbit, and no crossing was seen in 6 rabbits. After the injection of eserine (1.8 mgm.) reversible crossing was noted in 1 rabbit (fig. 2C). In the 2 animals that developed reversible crossing with prostigmin temporary crossed contractions had previously been elicited by asphyxia. Similarly, in the rabbit in which eserine caused crossed contractions previous crossings had been induced by acetylcholine and by block of the vagi.

B. *Acetylcholine.* Observations were made on 1 cat and 8 rabbits. In none of these animals did acetylcholine produce crossing when injected before administration of prostigmin or eserine. After injection of these drugs, however, acetylcholine (50 to 200 γ) caused transient crossed contractions in the 1 cat and in 7 of the 8 rabbits. In the cat and in 2 of the rabbits reversible crossing with acetylcholine after prostigmin (or eserine) occurred before and after denervation of the carotid sinuses (fig. 1B, C and fig.

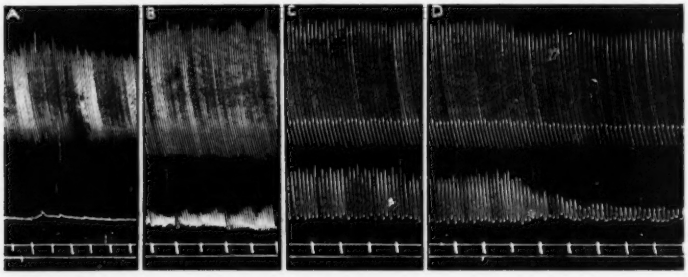

Fig. 3. Rabbit; dial. Left spinal semisection at C_2.
A, strychnine (0.2 mgm.); B, 7, and C, 90 minutes later; D, nembutal (6 mgm.).

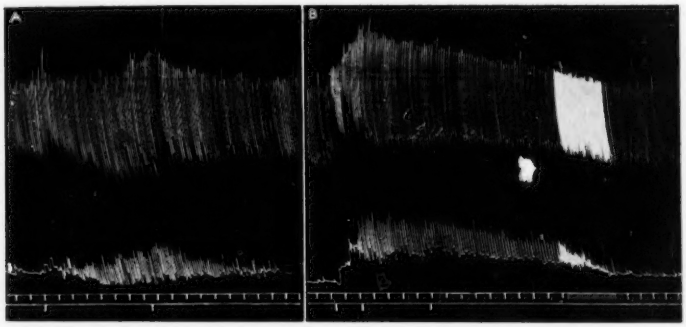

Fig. 4. Rabbit; dial; atropine (1 mgm. per kgm.); prostigmin (0.7 mgm. per kgm.). Left spinal semisection at C_2.
A, asphyxia; B, ether block of both vagi.

2A, B). The changes in rate and amplitude of the respirations at the time of crossing with acetylcholine were inconsistent (figs. 1 and 2).

C. *Strychnine.* Observations were made on 4 rabbits. In 2 a permanent crossing took place after injection of 0.2 to 0.5 mgm. per kgm.; injection of nembutal (6 mgm.) to one of these rabbits depressed only the crossed contractions (fig. 3). In another animal crossed effects did not occur until 6 mgm. of strychnine had been given. The fourth rabbit did not exhibit the crossed phenomenon with convulsant doses of strychnine;

crossed contractions appeared later after injections of prostigmin and acetylcholine.

D. *Section of the vagi.* Rosenblueth and Ortiz (*loc. cit.*) reported that in cats and rabbits crossing was not observed after section of the vago-sympathetic nerves (13 animals). In the present observations, made on 2 cats and 6 rabbits, permanent crossing was noted in 1 cat after this section. A similar permanent crossing was produced by vagal section in 1 cat and 4 rabbits which had received prostigmin previously. In 2 rabbits no crossing occurred on section of the vagi, but later permanent crossing was seen after section of the contralateral phrenic nerve. Two of the prostigminized rabbits in which there was permanent crossing when the vagi were cut, had shown reversible crossed contractions on block of these nerves by ether and by induction shocks (fig. 4B).

E. *Asphyxia.* Observations were made on 2 cats and 8 rabbits. Crossing with asphyxia was seen in the 2 cats and 6 of the rabbits (fig. 4A). In one of the remaining rabbits no crossing took place with asphyxia, with strychnine, section of the vagi, prostigmin, or acetylcholine, and crossing only appeared after section of the phrenic nerve. The other rabbit showed crossed effects after a large dose of strychnine.

Discussion. Rosenblueth and Ortiz (1936) showed that there are species differences with regard to the crossed phrenic phenomenon. They observed the phenomenon in dogs, cats, rabbits and woodchucks, but failed to find it in monkeys or guinea pigs. Section of the vago-sympathetic nerves produced crossed contractions in dogs (7 out of 8 animals). This procedure was at no time effective in cats or rabbits (13 animals). In our experience permanent crossing appeared in 1 cat on section of the vagi after a reversible crossing with asphyxia. Another cat and 4 rabbits, in which reversible crossed contractions had been produced by prostigmin and by acetylcholine protected by prostigmin, showed permanent crossing on section of the vagi. The only rabbit that developed permanent crossing after prostigmin had had the vagi cut previously. This indicates that after prostigmin cats and rabbits behave similarly to dogs on section of the vagi.

Crossing was observed after prostigmin, and after acetylcholine when protected by prostigmin, in several of the animals studied. Carotid sinus stimulation was ruled out by denervation of the carotid sinuses in 3 cases. Adrenal stimulation was shown to be of no importance by the failure of adrenaline to produce crossing. Strychnine produced crossing in 2 out of 4 animals. In 1 rabbit crossing did not occur with any of the drugs and section of the vagi, but crossed contractions followed section of the phrenic nerve. It appears, therefore, that section of the active phrenic is the most effective means of producing crossed contractions.

The crossings during asphyxia or after vagal section could be the result

of an increase (temporal or spatial) of the output from the respiratory center. During the crossings with prostigmin, acetylcholine and strychnine there was no evidence in most of the records of such an increased respiratory output. In these cases, therefore, the action was exerted at the neuronal mechanisms in the spinal cord concerned in the crossing. It is possible that these drugs may enhance transmission in the central synapses involved.

SUMMARY

In cats and rabbits under dial anesthesia, with spinal semisections at C_2 and consequent ipsilateral respiratory hemiplegia, crossed respiratory contractions of the paralyzed hemidiaphragm were produced or promoted by prostigmin or eserine (figs. 1 and 2), acetylcholine protected by prostigmin (figs. 1 and 2), strychnine (fig. 3), section of the vagi after prostigmin (figs. 1 and 4), and asphyxia (fig. 4).

The data emphasize that differences previously noted between rabbits, cats and dogs, with respect to the crossed phrenic phenomenon, are only quantitative.

The most efficient factor for producing crossed contractions is the section of the phrenic nerve (p. 105).

The authors wish to express their appreciation of the advice and encouragement given by Dr. A. Rosenblueth.

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THE SELECTIVE UPTAKE OF BROMINE BY THE THYROID GLAND WITH RADIOACTIVE BROMINE AS INDICATOR[1]

I. PERLMAN,[2] M. E. MORTON AND I. L. CHAIKOFF

From the Division of Physiology, University of California Medical School, Berkeley

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The ability of the thyroid gland to concentrate iodine has been clearly established by the early observations of Baumann, Marine and others (1) and more recently by investigations with radioiodine (2–5). Moreover, it has been shown recently that a large percentage of *normal circulating iodine* is *constantly* being removed by the thyroid gland. This was observed in studies in which "tracer"[3] amounts of radioiodine were introduced. As much as 65 per cent of the administered labeled iodine was removed by the thyroid gland in 24 hours (4).

This selective activity of the gland is not restricted to iodine, for it has recently been shown that the next higher halogen, ekaiodine, is retained somewhat similarly (6). These observations raised the question whether the next lower halogen, bromine, would also be stored in thyroid tissue. Although several attempts have been made to compare the bromine content of thyroid with that of other tissue, many of the older observations suffered from inadequate analytical procedures employed for the determination of bromine. More recently, however, Baumann, Sprinson and Marine have shown that the hyperplastic thyroid contains more bromine than the blood, whereas the resting colloid-containing gland has no more bromine than the blood (7).

EXPERIMENTAL. Radiobromine was produced by the neutron bombardment of bromobenzene containing 5 per cent by volume of aniline according to the method of Lu and Sugden (8). The bromobenzene-aniline solution was shaken with water, and the aniline was removed from the water layer by extraction with ether. In this manner a water solution of radiobromine containing very little bromine was obtained; it was estimated that each

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[2] Rosenberg Fellow in Physiology.

[3] A "tracer" dose of iodine refers to a sample of radioiodine containing no carrier. Such a sample of radioiodine contains only insignificant amounts of iodine (4).

millicurie of activity was accompanied by an amount of bromine of the order of magnitude of 10^{-6} mgm. Since each animal received less than 50 microcuries of radioactivity, the total bromine administered was negligible when compared with the amount present in the animal. In one experiment KBr was added in an amount that made the dose received by each animal equal to 0.12 mgm. of Br.

The bromine isotope used in this study was Br^{82}, which has a half-life of 34 hours (9). It was deemed advisable not to use Br^{80} (4.4 hr. half-life) because it undergoes an isomeric transition to Br^{80} with an 18 minute half-life (9). Before the radioactivity of any tissue was determined, Br^{80} was allowed to decay to a negligible concentration.

The solutions of radiobromine were administered intraperitoneally to rats and guinea pigs. At intervals thereafter the animals were anesthetized with nembutal. Blood samples were taken by heart puncture and the other tissues rapidly excised. All tissues were placed in large test tubes and thoroughly mashed by means of a stirring rod with an $AgNO_3$ solution (1 cc. of 0.15 M $AgNO_3$ per gram of tissue). After digesting this mixture on the steam bath for 1 hour, 3 cc. of concentrated HNO_3 were added for each gram of tissue and the heating continued for 8 hours. After diluting the digests with several volumes of water, the silver halides were filtered and mounted for the determination of radioactivity in the manner previously described for iodine (4). In order to equalize the mass of the precipitates of silver halides, 1 cc. of 0.02 M KBr was added in each case as carrier before digestion of the tissue was begun. This procedure was tested for recovery of bromine by the addition of radiobromine to inert tissue. The radiobromine was quantitatively recovered in all cases.

RESULTS. *Distribution of 0.12 mgm. of injected labeled bromine in the tissues of normal rats.* The content of radiobromine in tissues was measured at 2 intervals after the intraperitoneal injection of the sample of labeled bromine. The highest concentrations were found at both time-intervals in the *thyroid gland.* Thus at 3 hours after its introduction, 2 per cent of the administered labeled bromine was found in each gram of thyroid tissue, whereas 3 hours later 1.7 per cent was still present in each gram of this gland. Smaller amounts were found deposited in the liver, kidney, brain, salivary glands, pituitary and adrenals. Liver and kidney retained about equal amounts at the 2 time-intervals, namely, 0.7 to 0.8 per cent. Somewhat similar concentrations were found in the salivary glands and the pituitary. The lowest concentrations appeared in the brain and adrenal glands. Average values of 0.29 and 0.25 per cent were observed in the former, whereas each gram of adrenal tissue contained 0.37 and 0.31 per cent at 3 and 6 hours respectively. Whole blood contained higher amounts of the labeled bromine than all other tissues except thy-

roid. Thus at 3 and 6 hours after the administration of the labeled bromine 1.5 and 1.3 per cent of it was found in each gram of whole blood.

TABLE 1

The distribution of 0.12 mgm. of labeled bromine in the normal rat

(Each rat received intraperitoneally 1.5 cc. of a solution containing labeled bromine as KBr)

3 HOURS AFTER INJECTION				6 HOURS AFTER INJECTION			
Rat number*	Tissue	Br activity per gram†	Average	Rat number*	Tissue	Br activity per gram†	Average
8	Liver	0.95		11	Liver	0.62	
9	Liver	0.75		12	Liver	0.70	
10	Liver	0.63	0.78	13	Liver	0.71	
				14	Liver	0.69	0.68
8	Kidney	0.91		11	Kidney	0.78	
9	Kidney	0.89		12	Kidney	0.95	
10	Kidney	0.81	0.87	13	Kidney	0.71	
				14	Kidney	0.69	0.78
8	Brain	0.27		11	Brain	0.24	
9	Brain	0.28		12	Brain	0.28	
10	Brain	0.31	0.29	13	Brain	0.26	
				14	Brain	0.23	0.25
8	Whole blood	1.43		11	Whole blood	1.38	
9	Whole blood	1.53		12	Whole blood	1.11	
10	Whole blood	1.48	1.48	13	Whole blood	1.37	
				14	Whole blood	1.51	1.34
7, 8, 9, 10	Salivary glands‡		0.73	11, 12, 13, 14	Salivary glands		0.58
7, 8, 9, 10	Pituitary‡		0.65	11, 12, 13, 14	Pituitary		0.57
7, 8, 9, 10	Adrenals‡		0.37	11, 12, 13, 14	Adrenals		0.31
7, 8, 9, 10	Thyroid‡		2.02	11, 12, 13, 14	Thyroid		1.68

* Male rats weighing 200 grams were used.

† Refers to the per cent of the administered labeled bromine.

‡ Tissues pooled from 4 rats.

The distribution of a "tracer" dose of radiobromine in the tissues of normal guinea pigs and in guinea pigs treated with thyrotropic hormone. Twelve male guinea pigs weighing approximately 300 grams each were used in

this experiment. Six of these were treated with thyrotropic hormone[4] over a period of 10 days before the injection of radiobromine. Blood was taken by heart puncture after the animals had been anesthetized with nembutal. The blood was allowed to clot and serum removed by centrifugation. The gastrocnemius muscle was taken as the sample of muscle.

The highest concentrations of radiobromine were found in the thyroid gland of both normal and hormone-treated guinea pigs. This is particularly well shown at the 24-hour interval. The highest concentrations of radiobromine per gram of liver, muscle and adrenal gland did not exceed 0.32 per cent of the administered labeled bromine at the 2-hour interval,

TABLE 2

The distribution of a "tracer" dose of radiobromine in the tissues of normal guinea pigs and in guinea pigs treated with thyrotropic hormone†

TIME INTERVAL	ANIMAL NUMBER		THYROID WEIGHT		BROMINE ACTIVITY*											
					Thyroid				Adrenal		Liver		Muscle		Serum	
					Normal		Treated									
	Normal	Treated	Normal	Treated	Per gram	Whole organ	Per gram	Whole organ	Normal, per gram	Treated, per gram	Normal, per gram	Treated, per gram	Normal, per gram	Treated, per gram	Normal, per gram	Treated, per gram
hours			*gram*	*gram*												
2	1	7	0.045	0.152	0.68	0.031	0.880	0.134	0.234	0.178	0.112	0.167	0.114	0.111	0.489	0.561
2	2	8	0.055	0.108	0.52	0.029	0.816	0.0883	0.210	0.192	0.120	0.139	0.125	0.101	0.522	0.552
2	3	9	0.039	0.086	0.58	0.023	0.73	0.0629	0.191	0.321	0.120	0.170	0.104	0.141	0.506	0.704
24	4	10	0.040	0.091	0.52	0.021	0.614	0.056	0.148	0.141	0.102	0.0744	0.0706	0.0566	0.346	0.335
24	5	11	0.047	0.090	0.51	0.024	0.696	0.063	0.143	0.168	0.0855	0.0981	0.0742	0.0735	0.322	0.387
24	6	12	0.046	0.115	0.39	0.018	0.641	0.0737	0.138	0.139	0.0887	0.0844	0.0785	0.0670	0.362	0.358

* Refers to per cent of the administered labeled bromine.

† Each animal received subcutaneously 8 mgm. of thyrotropic preparation over a period of 10 days.

whereas the values per gram of thyroid tissues ranged from 0.52 to 0.88 per cent. At 24 hours the content of labeled bromine in liver, muscle and adrenal gland did not exceed 0.17 per cent per gram of tissue; at this time-interval thyroid contained from 0.40 to 0.70 per cent per gram.

No significant change in the uptake of labeled bromine by liver, muscle and adrenal gland resulted from treatments with thyrotropic hormone. Despite the fact that the thyroid gland showed a greater avidity for labeled bromine than the other tissues examined, hypertrophied glands produced

[4] We are indebted to Dr. Q. Bartz of Parke, Davis and Company, Detroit, for the thyrotropic preparation used in this investigation. This fraction assayed 4 guinea pig units per milligram. The unit is defined as the total dose in milligrams injected subcutaneously once daily for 4 days into 180 to 200 gram guinea pigs that produces on the fifth day minimal but definite hyperplasia of the thyroid in all of 6 animals.

by treatments with thyrotropic hormone failed to show striking increases in their uptake of bromine when compared with normal glands. Thus at the 24-hour interval the hypertrophied glands contained an average of 0.65 per cent of the administered labeled bromine per gram, as compared with 0.47 for normal glands. At the earlier interval the average values for normal and hypertrophied gland were respectively 0.59 and 0.81 per cent per gram of tissue.

COMMENT. It is generally accepted that administered chloride distributes itself principally, if not entirely, throughout the extracellular phase and can be used to determine the extent of these phases in a tissue.

TABLE 3

Calculation of extracellular phase based on distribution of labeled bromine

CONDITION	TIME	ANIMAL NUMBER	THYROID F	ADRENAL F	LIVER F	MUSCLE F
	hours					
Normal	2	1	123	43.8	20.2	20.6
	2	2	87.8	35.5	20.3	21.1
	2	3	101	33.2	20.9	18.1
Treated	2	7	138	28.0	26.2	17.5
	2	8	131	30.7	22.2	16.2
	2	9	91.5	40.2	21.3	17.7
Normal	24	4	132	37.7	26.0	18.0
	24	5	140	39.2	23.4	20.3
	24	6	95	33.6	21.6	19.1
Treated	24	10	162	37.2	19.6	14.9
	24	11	159	38.4	22.4	16.8
	24	12	158	34.2	20.8	16.5

Wallace and Brodie (10) compared $\frac{\text{tissue Br}}{\text{serum Br}}$ with $\frac{\text{tissue Cl}}{\text{serum Cl}}$ and concluded that the distribution of both halides is similar in all tissues examined except the brain. In the case of the brain, however, equilibrium is established with the halides in cerebrospinal fluid rather than with those in serum. It was concluded from these measurements that a selective retention of bromine by tissue cells is unlikely. Similar results were obtained by Weir and Hastings (11).

In table 3 are shown the values for the extracellular phase of tissues based on the distribution of radiobromine. The calculations were made according to Hastings and Eichelberger (12), the assumption being made for the present purpose that radiobromide distributes itself freely between

serum and the extracellular spaces only. The following equation was used in determining the extracellular phase of tissues:

$$F = \frac{Br_T \times 0.95 \times 0.92 \times 100}{Br_S \times 0.99}$$

where

F = Extracellular phase as grams per 100 grams of wet tissue,
Br_T = per cent of the administered labeled bromine recovered per gram of wet tissue,
Br_S = per cent of the administered labeled bromine recovered per gram of serum.

The figures 0.99 and 0.92 are values accepted for the fractions of water in the extracellular phase and serum respectively; 0.95 is the value for the Gibbs-Donnan ratio.

The average values for the extracellular phase of muscle, liver and adrenals obtained by this type of calculation are respectively 18.1, 22.1 and 36.0 grams per 100 grams of tissue. The results for muscle and liver agree satisfactorily with those of Weir and Hastings (11) and Wallace and Brodie (10). No values for the adrenals are available for comparison.

By this calculation, however, most of the values for extracellular phase, F, of thyroid tissue turn out to be greater than 100, i.e., greater than the entire mass of tissue. It is therefore apparent that the assumption upon which the F values for thyroid were determined is invalid. It may now be concluded that no simple relation exists between serum and thyroid bromine similar to that observed between serum and the other tissues examined.

SUMMARY

1. The uptake of bromine by various tissues of the rat and guinea pig was investigated with radioactive bromine as indicator.

2. The normal thyroid gland as well as the hypertrophied gland produced by injections of thyrotropic hormone showed a much greater uptake of labeled bromine than any of the other tissues examined. The content of radiobromine in the thyroid was too high to be explained by simple diffusion from serum.

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DEMONSTRATION OF VITAMIN A IN THE RETINA BY FLUORESCENCE MICROSCOPY

RUVEN GREENBERG AND HANS POPPER[1]

From the Department of Physiology and Pharmacology, Northwestern University Medical School and the Cook County Hospital and the Cook County Graduate School of Medicine, Chicago, Ill.

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Janeso and Janeso (1) described in a preliminary report the presence of green fading fluorescing droplets in the pigment coat of the light adapted eye of the albino rat, and the absence of such fluorescence from the dark-adapted eye. In accordance with the fluorescent microscopic study of Querner (2), they ascribed this fluorescence to vitamin A. Janeso and Janeso in correlating their findings with those of Wald (3) spoke of the reversible formation of vitamin A from visual purple.

The histological distribution of vitamin A in tissues of the albino rat has recently been more extensively studied (4, 5), and evidence was provided for the specificity of the green fading fluorescence in fixed tissue sections (for vitamin A); a short description of the retina of the light adapted eye was offered. In this paper we shall provide confirmation of the preliminary report of Janeso and Janeso, which, to our knowledge, has not yet been done.

METHODS AND MATERIALS. Under the fluorescence microscope a green fading fluorescence of lipoids is characteristic for vitamin A. The tissue is prepared by short formalin fixation. Frozen sections mounted in water are examined shortly after cutting.

The light is supplied by a mercury vapor bulb. The visible light is filtered out by a Corning Glass Filter no. 584, and a glass cell containing copper sulfate solution. An eyepiece filter cuts out the ultra-violet light after it has passed through the tissue.

Methylene blue staining does not significantly interfere with the fluorescence of vitamin A. Thus a methylene blue stained section can be examined both in visible and in ultra-violet light. This assists in the localization of the vitamin A fluorescence. A detailed description is given in a previous paper (4).

Light adapted eyes were prepared *in vivo* by placing the rat under a bright light for at least an hour. Dark adapted eyes were prepared *in vivo*

[1] Research Fellow of the Cook County Graduate School of Medicine.

by placing the rat in a dark room for at least two hours. Partially light adapted eyes were prepared by putting dark adapted eyes in light for a few minutes. The eyes of rats taken directly from the laboratory are referred to as laboratory adapted eyes.

The diets of the rats examined varied in their vitamin A content so that the rats were either completely deficient, partially deficient, normal or hypervitaminotic. Since 95 per cent of the vitamin A store is in the liver (6, 7), the nutritional state of the animal was evaluated by the vitamin A content of the liver as determined histologically and chemically.

One eye of each animal was examined histologically and the other macroscopically for visual purple content (colorimetric scale of Fredericia and Holm (8)).

Results. On gross inspection the retina of the *laboratory adapted eye* was usually brick colored. Microscopically the vitamin A fluorescence was seen in the pigment coat in small droplets. The droplets were arranged around the periphery of the pigment epithelial cells and connected by green fluorescing filaments. The vitamin A fluorescence faded within a few seconds upon irradiation. Thus the hexagon shaped cells were outlined until the vitamin A fluorescence had faded to change all the pigment coat to the uniform non-fading green of the cytoplasm. Only the large centrally located nuclei imparted no fluorescence.

In the examined frozen sections the retina was usually separated from the pigment epithelium except at the papilla of the optic nerve and at the ora serrata. The outer segments of the rod and cone layer were usually torn and remained within the pigment coat when the retina and pigment coat were separated.

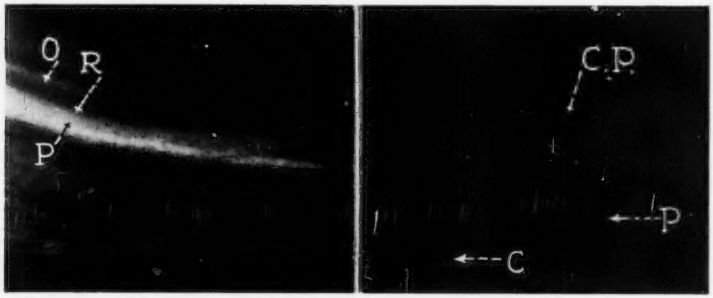
The rod and cone layer of the retina contained only minute traces of vitamin A. In addition, a green non-fading fluorescence was seen in the rod and cone layer and in the outer molecular layer of the retina. The nuclear layers were free of fluorescence. In the ciliary processes vitamin A fluorescence was seen in the interstitium: in the capillary endothelium and in the fixed connective tissue cells. The posterior portion of the ciliary body was rich in vitamin A fluorescence (fig. 1, A).

On gross inspection the retina of the *dark adapted eye* was deep red in color. Microscopically the vitamin A fluorescence of the pigment coat and of the rod and cone layer was either absent or only seen in traces. The green non-fading fluorescence was changed to a definite rust-brown color in the pigment coat, in the rod and cone layer, and in the outer molecular layer. The absence of droplets with vitamin A fluorescence from the pigment coat gave it a uniform appearance on contrast to the hexagonally outlined cells of the light adapted pigment coat. The vitamin A fluorescence of the ciliary body did not vary from that of the light adapted eye (fig. 1, B).

On gross inspection the retina of the *partially light adapted eye* was either pink or light red in color. Microscopically it was similar to the dark adapted eye except for a few traces of vitamin A fluorescence in the rust-brown fluorescing pigment coat.

The nutritional status of the rats did not significantly influence the fluorescence microscopic picture of the retina and the pigment coat. In vitamin A deficiency the vitamin A fluorescence might have been reduced but the fluorescence was never completely absent from the light adapted eyes; not even in the ulcerated eyes in extreme vitamin A deficiency (table 1).

In hypervitaminosis there was no apparent increase in the vitamin A fluorescence of the retina and pigment coat during light adaptation and the vitamin A fluorescence was absent during dark adaptation.



A B

Fig. 1A. Section of posterior wall of a light adapted eye of an albino rat. Vitamin A fluorescence localized in: *P*, the pigment coat, and *R*, the rod and cone layer. Non-fading green fluorescence in the outer molecular layer, *O*.

Fig. 1B. Section of dark adapted eye. Vitamin A fluorescence in the interstitium of the ciliary processes, *C. P.* The pigment coat, *P*, shows the dim non-fading rust-brown fluorescence. The blue fluorescence of the cornea, *C*, (appearing white in the black and white photograph) should not be confused with the vitamin A fluorescence.

The vitamin A fluorescence of the ciliary processes did depend upon the vitamin A nutritional status. It was absent in vitamin A deficient and hypovitaminotic animals. Its amount was increased in hypervitaminosis.

The vitamin A fluorescence and the green non-fading fluorescence of the light adapted eye and the rust-brown fluorescence of the dark adapted eye were not changed by formalin fixation (for not more than 10 hrs.) or by *in vitro* treatment with hydrogen peroxide, sodium hydrosulfite (as reducing agent), or normal ammonium hydroxide. Alcohol and acetone removed the vitamin A fluorescence and the non-fading green and yellow-brown fluorescence of pigment coat and retina. Dilute hydrochloric acid and glycerine left the vitamin A fluorescence unchanged, but with the former

the non-fading green and rust-brown fluorescence was made even brighter while with the latter the non-fading green and rust-brown fluorescence was changed to a red.

The cornea, lens and the other tissues of the eye did not show vitamin A fluorescence except for the adjacent fat cells which did show it in accordance with the nutritional status.

Discussion. There are two types of vitamin A distribution in the eye: 1. In the ciliary body the vitamin A fluorescence is seen in the interstitial elements such as the connective tissue cells and the capillary endothelium; the distribution is similar to that of the meninges, serous membranes, lungs, etc. (5), and the fluorescence reflects the nutritional status of the animal. 2. In the pigment coat and in the retina there is a specific vitamin A distribution, which is practically independent of the nutritional status. It is instead dependent upon the state of light adaptation as described by

TABLE 1

Vitamin A fluorescence of the pigment coat and rod and cone layer of the retina of 86 albino rats under varied nutritional conditions and under varied states of light adaptation

VITAMIN A FLUORESCENCE IN THE RETINA	VITAMIN A FLUORESCENCE OF THE LIVER							
	Absent		Traces		Normal		Hypervitaminotic	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Laboratory adapted eyes	8		20		10		5	
Light adapted eyes	2				7		4	
Partially light adapted eyes	2				5			
Dark adapted eyes		8		2		9		4

Janeso and Janeso. Possibly there are reduced amounts in vitamin A deficiency; but complete absence of vitamin A has never been seen by us. Hemeralopia, then, is not the result of a complete absence of vitamin A. A definite reduction of vitamin A is not necessarily seen histologically. These findings therefore neither affirm nor contradict the adaptometric studies.

The vitamin A in the second location depends upon the functional state. According to Wald it is formed from the visual purple of the outer segments of the rods in light adaptation. There is an intermediate state during which the visual purple is changed to visual yellow in a reversible reaction. The visual yellow is broken down to colorless end-products, among which is vitamin A. The vitamin A is in part used again in the slow transformation back to visual purple.

The histological studies are in agreement with Wald's cycle in that the vitamin A fluorescence is seen in light adaptation in concentrations de-

pending upon the degree of light adaptation; in that it is absent in complete dark adaptation; in that by far the strongest fluorescence is seen in the pigment coat and not in the rod and cone layer. Grossly these eyes showed high amounts of visual purple in the retina in dark adaptation, lesser in partial adaptation and none in light adaptation. Whether or not the non-fading rust-brown fluorescence is due to visual purple or visual yellow cannot be determined.

The visual purple or visual yellow droplets demonstrated in visible light (9, 10, 11) were not seen in fluorescence microscopy. We saw visual purple macroscopically (in accordance with Holm, 12) and vitamin A histologically in severe vitamin A deficiency contrary to Tansley (13) and Johnson (14) who report its absence.

CONCLUSIONS AND SUMMARY

There are two types of vitamin A distribution in the eye as shown by fluorescence microscopy, namely: *a*, in the ciliary processes where it depends on the nutritional status and where it is independent of the state of light adaptation; and *b*, in the pigment coat and in the rod and cone layer of the retina where it is functional. Here the presence of vitamin A does not depend on the nutritional state. Possibly there is a reduction in amount in vitamin A deficiency. As described by Jancso and Jancso the presence of vitamin A depends on the state of light adaptation. It is absent in dark adaptation. These observations are in agreement with Wald's cycle describing the rôle of vitamin A in vision.

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WEIGHT CHANGES IN THE CORTEX AND THE MEDULLA OF THE ADRENAL GLAND OF THE DOG IN ACUTE VITAMIN-B_1 DEFICIENCY

JULIA E. GOODSELL

From the Department of Physiology, University of Chicago, Chicago, Illinois and the Department of Physiology, University of Washington, Seattle

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Numerous investigators have shown that there are many pathological conditions in which the adrenals hypertrophy while other endocrine glands atrophy. Outstanding among the conditions in which this enlargement is reported to occur are injections of hormones, avitaminoses, inanition and infection (1–6). Histological changes in both the cortex and medulla have been described, but there is no clear cut evidence indicating whether these changes consistently involve the cortex, the medulla or both. From the variety of changes which have been reported it seems probable that the part of the gland affected may depend upon either the nature or the severity of the pathological condition. Evidence for this is given by Deansley (6) who made a study, not only of the histological picture, but also of the volume changes in the cortex and medulla of the adrenals in mice when given injections of thyroxine, morphine or killed B. gaertner.

Although many workers have reported enlargement of the adrenals in vitamin-B_1 deficiency, data are lacking in regard to the extent of the involvement of each part of the gland. Sure (4) has reported a total enlargement of the adrenals in rats in vitamin-B_1 deficiency which was 50 per cent greater than that of the controls kept at the same plane of nutrition.

In this study an attempt has been made to determine weight changes in both parts of the gland in acute vitamin-B_1 deficiency in the dog so that the portion responsible for the increased weight may be known.

METHODS. Seven young mature dogs weighing from 5 to 14 kgm. were used. Each dog was wormed, immunized against distemper, and maintained on a normal diet for at least one week before the deficient ration was started. This ration, which was fed until acute symptoms of vitamin-B_1 deficiency appeared, consisted of:

	Per cent
Casein	19
Sucrose	60

	Per cent
Cottonseed oil	8
Salt mixture (186)*	4
Autoclaved yeast	8
Agar	1
Cod liver oil concentrate†	

* Manganese and copper added.
† Squibbs Adex Tablets—1 tablet per day.

The ration was given ad libitum, the average daily consumption during the first week on the diet being 150 grams. After this time the food consumption became more variable. Five to six days before the appearance of acute symptoms the food intake averaged approximately 30 grams. Table 1 shows the initial and final body weights of each dog and the time required to produce severe symptoms of polyneuritis in each case (36–59 days). Prior to the onset of acute symptoms frequent attacks of vomiting occurred. Three to four days later the dogs became extremely spastic and presented the characteristic symptoms of acute B_1 deficiency. All dogs were allowed to live 24 hours after the appearance of these symptoms, then killed, and the adrenals removed immediately.

Determination of weights of the cortex and medulla. In order to save the cortex of the glands for physiological tests the following procedure was devised for determining the weights of the cortex and the medulla. Each pair of glands was dissected free of connective tissue and fat in a moist chamber and weighed in a weighing bottle. Each gland was cut in half longitudinally and the medulla, which stood out clearly from the cortex, was carefully teased out by blunt dissection. All of the adrenal glands removed from the vitamin-B_1 deficient dogs as well as from the controls were weighed in this manner (tables 1 and 2).

To establish the range of variation of these ratios in normal dogs, the adrenals from 96 dogs obtained from the pound were dissected and weighed. Each dog was classified as to approximate age (immature, mature, or old), sex, general health, and, in the case of females, its stage in the estrual cycle. All dogs were weighed immediately after being killed.

Baker (7) has shown that the weight of the adrenal glands varies with the age and the weight of the animal and that the ratio of medulla weight to cortex weight is approximately the same in mature males as in females in dioestrus. Hence, as a basis for comparison, only those dogs were chosen that were healthy, mature, in dioestrus, in the case of females, and within the same weight range as those in the series of B_1 deficient animals (table 2). All of the data were analyzed statistically for significance of difference, using a formula for small samples given by Tippett (8).

Results and discussion. A common method used to indicate the size of an organ is to express it as a per cent of the body weight. Com-

parison of the percentage organ weights for the vitamin-B_1 deficient animals with those for normal animals will indicate whether any change in size has occurred. As a loss of body weight usually accompanies vitamin-B_1 deficiency, the question arises whether the initial or the final body weight should be used in making these computations. Even though there is no actual change in size of an organ, if a marked loss of body weight occurred during the experimental period, the values obtained by expressing the organ weight as a per cent of the *final* body weight will be larger than those for normal animals.

As McCarrison (1) and Jackson (5) have shown that the loss of body weight in the various avitaminoses is due not only to a loss of body fat but also to atrophic changes in the organs and tissues, it should be expected that a loss of weight of the endocrine glands would also occur. Hence, the

TABLE 1

Showing weights of adrenal glands in relation to body weight in vitamin-B_1 deficient dogs

DOG NUMBER	SEX	DEPLETION TIME	INITIAL BODY WT.	FINAL BODY WT.	GLAND WT.	CORTEX WT.	MED. WT.	GLAND WT. PER CENT INITIAL BODY WT.	GLAND WT. PER CENT FINAL BODY WT.	CORTEX WT. PER CENT INITIAL BODY WT.	CORTEX WT. PER CENT FINAL BODY WT.	MED. WT. PER CENT INITIAL BODY WT.	$\frac{\text{CORTEX WT.}}{\text{MED. WT.}}$
		days	*kgm.*	*kgm.*	*gm.*	*gm.*	*gm.*						
1	F	36	7.26	8.35	0.922	0.856	0.066	0.013	0.011	0.012	0.010	0.0010	13.0
2	M	37	7.09	6.40	0.901	0.826	0.081	0.013	0.014	0.012	0.013	0.0011	10.2
3	M	39	5.38	5.87	0.941	0.895	0.046	0.017	0.016	0.017	0.015	0.0010	10.8
4	M	48	7.79	6.46	1.367	1.256	0.111	0.017	0.021	0.016	0.019	0.0014	11.3
5	M	49	8.87	6.89	0.991	0.902	0.089	0.012	0.014	0.010	0.013	0.0010	10.1
6	M	59	10.4	7.83	1.207	1.082	0.125	0.012	0.015	0.011	0.014	0.0012	8.6
7	M	55	13.9	8.49	1.649	1.521	0.128	0.012	0.019	0.011	0.018	0.0010	11.8

maintenance of the weight of a gland under conditions in which other organs are losing weight can be considered a *relative* hypertrophy. On the other hand, the hypertrophy can be considered as *absolute* if the weight of the gland, when expressed as a per cent of the *initial* body weight, is greater than that for normal animals.

In order to take both of these factors into consideration computations were made both on the basis of the initial and of the final body weights (table 3). It can be seen that the increase in size of the gland is due to the cortex, which undergoes an absolute (3A) as well as a relative (3B) hypertrophy.

On the other hand, the medulla weights of the adrenals of the B_1 deficient dogs, when expressed as a per cent of the initial body weight are lower than those for the normal animals, showing a tendency for this part of the gland

to follow the same trend as most of the organs and tissues (table 3A). For this reason the atrophy would not be apparent if the medulla weight

TABLE 2

Showing variation in weight of adrenal glands in relation to body weight in normal mature dogs

DOG NUMBER	SEX	BODY WT.	GLAND WT.	CORTEX WT.	MED. WT.	GLAND WT. PER CENT BODY WT.	CORTEX WT. PER CENT BODY WT.	MED. WT. PER CENT BODY WT.	CORTEX WT. / MED. WT.
		kgm.	*gm.*	*gm.*	*gm.*				
1	M	7.27	0.962	0.815	0.147	0.013	0.011	0.0021	5.5
2	M	8.60	1.060	0.929	0.131	0.011	0.011	0.0013	7.1
3	M	9.30	1.053	0.878	0.175	0.011	0.009	0.0019	5.0
4	M	12.70	1.190	0.988	0.202	0.009	0.008	0.0016	4.0
5	M	7.97	1.70	1.320	0.387	0.021	0.016	0.0040	3.4
6	M	12.20	1.510	1.340	0.176	0.012	0.015	0.0014	7.6
7	M	12.0	1.320	1.116	0.204	0.011	0.009	0.0017	5.5
8	M	8.80	0.903	0.689	0.214	0.010	0.008	0.0023	3.4
9	M	8.30	0.993	0.838	0.055	0.012	0.010	0.0066	15.1
10	M	7.27	1.146	0.991	0.155	0.016	0.014	0.0021	6.4
11	M	11.13	0.909	0.767	0.142	0.008	0.006	0.0014	5.4
12	M	7.38	0.789	0.722	0.067	0.010	0.010	0.0009	10.7
13	M	7.72	1.045	0.941	0.104	0.013	0.012	0.0013	9.3
14	M	11.37	0.954	0.817	0.137	0.008	0.007	0.0012	5.9
15	M	11.30	0.792	0.705	0.087	0.007	0.006	0.0008	8.1
16	M	9.10	0.715	0.635	0.080	0.008	0.009	0.0009	7.9
17	M	12.05	1.110	0.965	0.145	0.009	0.008	0.0012	6.6
18	M	12.50	1.220	1.030	0.190	0.010	0.008	0.0015	5.4
19	M	13.0	1.362	1.217	0.145	0.010	0.009	0.0011	8.4
20	M	12.50	1.169	0.968	0.201	0.009	0.008	0.0016	4.8
21	F	6.20	0.659	0.571	0.088	0.010	0.009	0.0014	6.5
22	F	9.70	1.219	1.076	0.143	0.013	0.011	0.0015	7.5
23	F	8.60	0.935	0.848	0.087	0.011	0.010	0.0010	9.8
24	F	8.80	1.30	1.113	0.187	0.015	0.013	0.0021	6.0
25	F	5.90	0.705	0.531	0.174	0.012	0.009	0.0030	3.3
26	F	7.72	0.950	0.786	0.164	0.012	0.010	0.0021	4.8
27	F	7.50	0.932	0.786	0.146	0.012	0.010	0.0020	5.4
28	F	6.58	1.044	0.879	0.165	0.016	0.013	0.0025	5.3
29	F	9.09	1.382	1.197	0.185	0.015	0.013	0.0020	6.5
30	F	6.80	1.180	0.982	0.199	0.017	0.014	0.0030	4.9
31	F	10.11	1.029	0.915	0.114	0.010	0.009	0.0011	8.0
32	F	8.40	1.115	0.930	0.184	0.013	0.011	0.0022	5.0
33	F	11.37	1.012	0.878	0.134	0.009	0.008	0.0012	6.6

were expressed as a per cent of the *final* body weight, as the loss in total body weight would mask the loss in weight of the medulla.

An increase is also noted in the ratio of the weights of cortex to medulla

(table 3B). Baker (7) from a comprehensive study of the weights of the adrenals of 1250 dogs reported that the weights of cortex to medulla were approximately 5 to 1 in both sexes. The results for normal dogs obtained in this study showed average ratios of 6 to 1. Considering the comparatively crude method employed, that the cortex might be saved for further work, this ratio corresponds well with Baker's. In the B_1 deficient dogs the average ratio for weight of cortex to medulla was 10.8 to 1 which is significantly higher than that obtained for the normal dogs. It can be seen that the increase in the ratio could be due either to an increase in cortex weight or to a decrease in medulla weight. From the results shown in table 3B it seems probable that both factors are involved.

TABLE 3

Comparison of adrenal glands of normal and of vitamin-B_1 deficient dogs

A. Expressed as per cent initial body weight

	NUMBER OF DOGS	GLAND WEIGHT PER CENT	STAN. DEV.	t	p	CORTEX WEIGHT PER CENT	STAN. DEV.	t	p	MED. WEIGHT PER CENT	STAN. DEV.	t	p
B_1 def.	7	0.014	0.0024	2.41	0.04	0.0125	0.0024	2.42	0.04	0.0011	0.0002	2.4	0.04
Normal	33	0.011	0.0025			0.0101	0.0023			0.0017	0.0006		

B. Expressed as per cent final body weight

	NUMBER OF DOGS	GLAND WEIGHT PER CENT	STAN. DEV.	t	p	CORTEX WEIGHT PER CENT	STAN. DEV.	t	p	CORTEX / MEDULLA	STAN. DEV.	t	p
B_1 def.	7	0.016	0.0030	4.0	0.01	0.0145	0.0028	4.2	0.01	10.8	1.29	5.1	0.01
Normal	33	0.011	0.0025			0.0101	0.0023			6.0	2.36		

As the results presented above indicate that the cortex is responsible for the enlargement of the adrenal glands in vitamin-B_1 deficiency in the dog, the question arises whether this necessarily means that there is an increased secretory activity of this part of the gland. Some workers have reported histological changes in the cortex under various pathological conditions which they have interpreted as an indication of increased activity. But as yet there is no direct evidence to justify this assumption. In a paper to follow, an attempt has been made to correlate weight changes of the cortex in vitamin-B_1 deficiency with changes in activity as indicated by a biological test.

SUMMARY

1. The adrenal cortex undergoes both a relative and an absolute hypertrophy in acute vitamin-B_1 deficiency in the dog.

2. The adrenal medulla shows a tendency to atrophy.

3. The ratio of the weight of cortex to medulla in normal dogs was found to be 6 to 1 whereas in the vitamin-B_1 deficient dogs it was 10.8 to 1.

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CHANGES IN CONCENTRATION OF STEROID COMPOUNDS IN THE ADRENAL CORTEX OF THE DOG IN VITAMIN-B_1 DEFICIENCY AS INDICATED BY THE BITTERLING TEST

JULIA E. GOODSELL

From the Department of Physiology, University of Chicago, Chicago, Illinois and the Department of Physiology, University of Washington, Seattle

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One of the outstanding changes described in the adrenal cortex in vitamin-B_1 deficiency is the change in the distribution of lipoids. McCarrison (1) and Kellaway (2), on the basis of histological studies, reported a wider distribution of the lipoids in the cortex in avian beri beri. Findlay (3) found a similar picture in the rat. That this change might conceivably be related to functional activity seems probable, as the various compounds which have been isolated from the cortex and shown to maintain life in an adrenalectomized animal are steroid compounds and would therefore be contained in the lipoid fraction of the gland. Zwemer (4), who made a study of adrenal morphology in various animals under both normal and pathological conditions, relates the wider distribution of lipoids to increased activity of the gland. Hoerr (5), on the other hand, does not think there is any good morphological evidence for indicating the function of the adrenal lipoids. Whitehead (6, 7) has shown that species variation must be considered in interpreting changes in cortical lipoids, and that the distribution changes with sex and age. As it is debatable whether changes in morphology of the gland should be used to gauge its functional state a physiological approach to the problem seemed desirable.

In 1936 Barnes, Kantner and Klawans (8) reported that aside from the gonads the adrenal glands were the only tissue which would yield an extract causing a lengthening of the ovipositor of the Japanese bitterling. Other investigators have shown that this reaction can be produced at will by various androgens and estrogens, and also by pure corticosterone (9 to 13). These results suggest that the bitterling test, though not specific for any one steroid compound, can be used to indicate the presence of this particular group of compounds. As this test can be made roughly quantitative it might serve as a useful criterion of the functional state of the adrenal glands until the identity of the true cortical hormone (or hormones) has been established.

METHODS. A. *Measurement of ovipositor lengths.* Kleiner, Weisman

and Mishkind (14) and DeWit (11, 15) determined the extent of elongation of the ovipositor of the bitterling by visual comparison with the length of the rays of the anal fin. Since the ovipositor may elongate to a distance two or three times the length of these rays this method is only suitable for detecting gross changes. In order to make the more accurate measurements necessary for quantitative study, an objective record of ovipositor lengths was obtained by photographing the ovipositor against a millimeter ruler at constant position and magnification (2×). This was done by holding the fish in a glass compartment against the side of a square glass container. The negatives were projected onto a sheet of paper (enlargement 100×), and the outline of the ovipositor traced. This outline was then marked off with a marking wheel and the ovipositor length recorded in terms of the number of spacings made by the wheel. By photographing the ovipositor of one fish ten times and measuring each negative by this method the standard deviation was 4.3 (based on an average of 131 spacings).

To eliminate the element of variability in initial ovipositor lengths all data are expressed as per cent lengthenings over the initial lengths; in each case the initial length was determined prior to placing the fish in the solution. During the experimental period the length was determined at approximately 12-hour intervals. By using a running-water bath the temperature was maintained between 17° and 19°C. throughout the time these experiments were carried out (Jan. to May, 1940).

B. *Preparation of lipoids from the adrenal cortex.* The glands used to test the bitterling response were obtained from the dogs of the previous investigation, on which measurements of the weight changes in acute vitamin-B_1 deficiency had been made. The cortex of the gland, which had been dissected away from the medulla, was thoroughly ground with ether at room temperature. Ten cubic centimeters of ether per gram of tissue was used and the grinding continued for ten minutes. This procedure was carried out three times to insure complete extraction of the lipoids. Aliquots representing known amounts of cortex were pipetted into liter beakers. After evaporating to dryness the extract was suspended in 100 cc. of water at 55°C. and the solution made up to 500 cc. with water from the aquarium. Two fish were placed in each beaker after the solutions had reached the temperature of the water in the aquarium.

C. *Standardization of the bitterling response.* In order to establish differences in the concentration of the active compounds in different glands it was necessary to work below the concentration yielding a maximal lengthening. Barnes et al. (8) reported that the extract from 0.75 to 1.0 gram of adrenal cortex per liter of water was necessary to produce lengthening of the ovipositor. In some preliminary experiments, performed during the fall months to check their results, it was found that a concentration of

less than a gram of cortex per liter of water failed to produce consistent lengthening in all of the fish treated. Consequently this was the lowest concentration which could be used at this time. In order to determine whether concentrations greater than 1:1000 would produce proportionately greater lengthening, solutions were prepared with the aliquots representing one, two and three grams of cortex, each suspended in 1000 cc. of water. Four fish were treated with each concentration. It can be seen from figure 1 that there is less difference between the 3:1000 and the 2:1000 concentrations than between the 2:1000 and the 1:1000 concentrations. Thus the 3:1000 is approaching the concentration that will produce a maximal response. With the 1:1000 concentration not only is the total lengthening less but also a regression of the ovipositors begins at the end of 48 hours. No regression is noted in the ovipositors of the fish treated with the two higher concentrations even at the end of 66 hours. Treatment was stopped after this period of time because previous experiments had shown that if the fish were left longer in solutions of high concentrations, many of them died. On the basis of these observations the 1:1000 concentration was considered sufficiently below the maximal concentration and was used in the experiments performed during the month of February, 1940.

As Kleiner et al. (9) reported a change in sensitivity of the fish with the approach of the breeding season (March to August), a second standardization of the bitterling response was made in March, 1940, before beginning the assay of adrenal cortical steroids from the second group of dogs. Concentrations of 1:1000 and 1:2000 were used, six fish being treated with each concentration. Since these lower concentrations were not injurious to the fish, treatment was continued for 90 hours in order to obtain a more complete picture of the regression of the ovipositors. The results given in figure 2 show that the 1:2000 concentration produced a lengthening of the ovipositors of approximately the same magnitude as the 1:1000 concentration had produced in the previous standardization. And again there were differences both in the per cent lengthening of the ovipositors and in the time at which regression occurred. Since the 1:1000 concentration produced considerably greater elongation in this month than in the preceding month it was thought advisable to use the lower concentration in subsequent experiments.

In assaying the cortical extract from the normal and the vitamin-B_1 deficient dogs the per cent lengthenings of similar groups of fish treated during the same time interval were compared. This seemed preferable to comparing responses of the same group of fish treated first with extract from normal dogs and then a month later with that from B_1 deficient animals for the following reasons: 1, no fish could be treated oftener than once a month, as previous experiments had shown less consistent responses when tests were run at more frequent intervals; 2, the death of any of the

fish would alter the character of the groups, and 3, the change in age and condition of the fish would cause changes in sensitivity to the extract.

RESULTS. *Group I.* In February, 1940, 11 fish were treated with the extracts of the adrenal cortex from three dogs in which acute symptoms of vitamin-B_1 deficiency had been produced (table 1). During the same month, ten fish were treated with extracts from the adrenals of normal dogs. In figure 3 the average per cent lengthenings of the groups of fish are plotted against time. Approximately a 43 per cent increase in ovipositor lengthening is noted in the fish treated with the extract from the adrenals of the B_1 deficient dogs over that obtained in the fish treated with the extract from the glands of normal dogs.

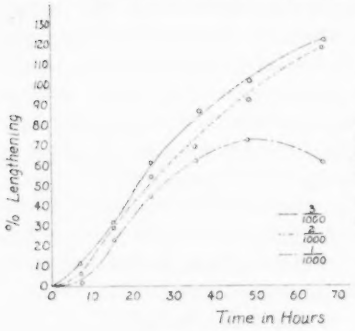

Fig. 1

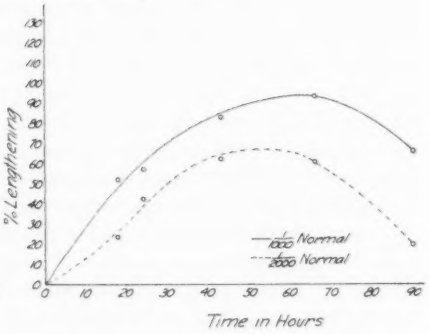

Fig. 2

Fig. 1. Bitterling response, February, 1940, to different concentrations of extract of adrenal cortex from normal dogs.

Fig. 2. Bitterling response, March, 1940, to different concentrations of extract of adrenal cortex from normal dogs.

Group II. In March, 1940, the responses of 15 fish treated with extracts from the adrenals of two dogs suffering from acute vitamin-B_1 deficiency were compared with those of 20 fish treated with extracts from the glands of normal dogs (fig. 4). Again, there is a much greater lengthening of the ovipositors of the fish treated with the extract from the adrenals of the B_1 deficient animals (about 50 per cent greater lengthening than in the controls).

Group III. Eighteen fish were treated with the extracts from the adrenals of two B_1 deficient dogs at the end of April, 1940. During the same time interval 17 fish were treated with the extracts from the glands of normal dogs. Results are shown in figure 5. The difference between the maximal per cent lengthening is not as great as in the two former groups (10 per cent). The smaller difference may have resulted from a further change in sensitivity of the fish due to approach of the breeding

season. This theory is borne out by the progressive increase in per cent lengthening of the ovipositors of the fish treated with the extract from the glands of normal dogs in successive months (table 1). If the sensitivity had increased, the concentration of 1:2000 of the extract from the adrenals of normal dogs was not sufficiently below that concentration necessary to evoke a maximal response and may even have been sufficient to produce maximal responses in some of the fish. However, the onset of regression of the ovipositors was later than in the controls, a factor which was shown by the standardization to vary with the concentration of extract used, and one which can be used to make reliable comparisons.

COMMENT. In analyzing the data statistically two factors have been considered: 1, the maximum per cent lengthening, and 2, the per cent

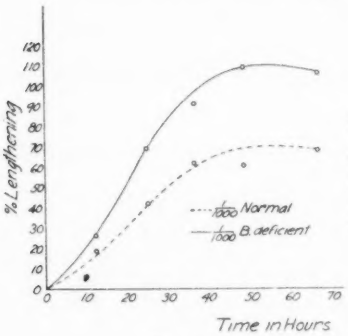

Fig. 3

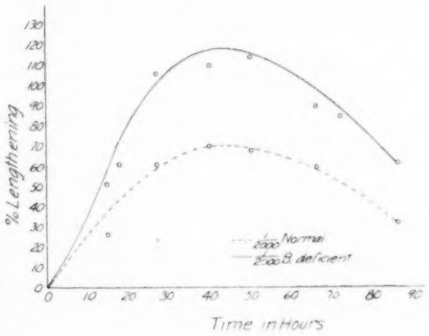

Fig. 4

Fig. 3. Bitterling response, group I, February, 1940, to 1:1000 concentration of adrenal cortical extracts.

Fig. 4. Bitterling response, group II, March, 1940, to 1:2000 concentration of adrenal cortical extracts.

lengthening at the end of 60 hours. Analysis of the results (Tippett, 16) based on the maximum per cent lengthening of the ovipositors shows that in groups I and II there are significant differences between the responses of the fish treated with the extract from the adrenals of the B_1 deficient dogs and those of the fish treated with the extract from the adrenals of normal dogs (table 1). In group III, which was run during April, the ten per cent difference over the normal in the maximum per cent lengthening of the ovipositors is not great enough to be statistically valid.

The second comparison, of the per cent lengthening of the ovipositors at the end of 60 hours, was chosen because regression of the ovipositors had begun in all of the fish by this time. In groups I and II this analysis gives almost identical results with those of the preceding method. By employing this method of analysis it is possible to validate the differences in the third group statistically (table 1). Thus a significant change in the

steroid compounds in the third group has been demonstrated in spite of the increased sensitivity of the fish. Due to a scarcity of fish it was impossible to carry out a third standardization at this time.

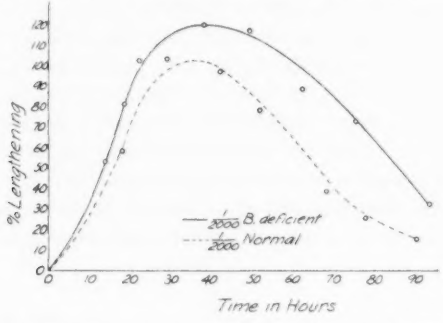

Fig. 5. Bitterling response, group III, April, 1940, to 1:2000 concentration of adrenal cortical extracts.

TABLE 1

Comparison of effects on bitterling response of extract of adrenals from normal and from vitamin-B_1 deficient dogs

GROUP	NUMBER OF DOGS	DEPLETION PERIOD	MONTH TREATED	CONCENTRATION OF SOLUTION	NUMBER OF FISH	MAXIMUM PER CENT LENGTH	STAN. DEV.	t	p	PER CENT LENGTH AT 60 HOURS	STAN. DEV.	t	p
		days											
I													
B_1 def.	3	37											
		38	Feb.	1:1000	11	115.0	19			105.0	26		
		40						4.0	0.01			3.6	0.01
Normal	2	0	Feb.	1:1000	10	79.0	19			69.0	15		
II													
B_1 def.	2	48											
		49	Mar.	1:2000	15	121.0	38			96.0	41		
								3.5	0.01			3.3	0.01
Normal	4	0	Mar.	1:2000	20	81.3	25			58.0	29		
III													
B_1 def.	2	55											
		59	Apr.	1:2000	18	126.0	27			89.0	38		
								1.0	0.3			2.68	0.02
Normal	3	0	Apr.	1:2000	17	116.0	34			57.0	29		

Discussion. As it has been demonstrated that the various steroid compounds isolated from the adrenal cortex vary considerably in respect to their ability to maintain the life of adrenalectomized animals, there is

the possibility that the greater potency of the extract from the adrenals of the B_1 deficient dogs may be due merely to a shift from a less active to a more active compound. However, from the results obtained in this investigation it seems most probable that the greater effect is due to an increase in concentration of these compounds in the adrenal cortex. The results from the standardization of the bitterlings showed that increased amounts of an identical extract produced greater elongation of the ovipositors. This would seem to be a concentration effect. That the effect is due to an increased concentration of the steroid compounds is also supported by histological evidence in which an increased lipoid content and a wider distribution of the lipoids throughout the cortex have been described. Finally, it has been shown (Goodsell, 1941) that the hypertrophy of the adrenal gland which occurs in acute vitamin-B_1 deficiency is due to an increase in size of the cortex. The results from this study demonstrate an accompanying increased potency of the steroid fraction. The simplest explanation of these related phenomena seems to be that an increased amount of the active substance has been produced.

SUMMARY

1. The use of the bitterling test as a sensitive biological method for a quantitative determination of the changes in the concentration of the steroid compounds in the adrenal cortex has been described.

2. By the use of this test it has been shown that the concentration of steroid compounds in the adrenal cortex was increased in dogs suffering from acute vitamin-B_1 deficiency.

Acknowledgment is made of indebtedness to Dr. A. J. Carlson, under whose direction this work was carried out, and to Dr. A. W. Martin for his constructive advice and criticisms.

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THE INFLUENCE OF FATS ON THE MOTOR ACTIVITY OF THE PYLORIC SPHINCTER REGION AND ON THE PROCESS OF GASTRIC EVACUATION STUDIED BY THE BALLOON-WATER MANOMETER AND BY THE OPTICAL MANOMETER-FLUOROSCOPIC TECHNICS[1]

J. P. QUIGLEY, J. WERLE, E. W. LIGON, JR.[2], M. R. READ, K. H. RADZOW AND I. MESCHAN

From the Department of Physiology, Western Reserve University Medical School, Cleveland, O.

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Fats introduced into the lumen of the proximal intestine inhibit the *body* of the stomach of fasting animals (1, 2). Extending these studies, we have employed the tandem balloon method of Meschan and Quigley (3) to make a qualitative study of the effects of fats on the motor activity of the pyloric *sphincter region* (antrum, sphincter, duodenal bulb and distal duodenum). In a second series of experiments, a modification of the optical manometer-fluoroscopic technic of Brody, Werle, Meschan and Quigley (4) was used to measure quantitatively the effects of fats on antral and bulbar pressures. Our recording tips for these studies were placed in the antrum and bulb on either side of the sphincter 18 mm. apart, and were made of soft rubber with metal inserts. The internal diameter of the antral and bulbar tips was respectively 5 and 3.5 mm.

Fasting animals. The test substances at body temperature were introduced into the duodenum of dogs 18 hours post-cibal at the onset of the experiment. The pressure employed for injection (15 cm. of water) just sufficed to overcome the friction in the catheter and cause the material to enter the duodenum slowly but without distending it. Time intervals in this report were measured from the *beginning* of the fat administration period.

Cream having 25 to 30 per cent butter fat and egg yolk with approximately 33 per cent lipids were employed as common examples of naturally emulsified fats. In 75 experiments cream and in 10 experiments egg yolk was administered into the distal duodenum of animals being studied by

[1] This investigation was supported in part by a research grant from the Ella Sachs Plotz Foundation.

[2] Department of Pharmacology, the George Washington University, School of Medicine, Washington, D. C.

the tandem balloon method. Inhibition of motility occurred in the *entire* sphincter region. This was manifested by a decrease in frequency and amplitude of the recorded waves which was most pronounced in those from the antrum and sphincter, less marked in the bulbar and comparatively slight in the distal duodenal records (fig. 1). With 10 cc. doses of either cream or egg yolk, the inhibition began in 1.5 to 2 min. and became complete in 4 min. in the antrum and sphincter; if inhibition of the bulb became complete, it developed in 7 min. Diminution of tone, as indicated by a decrease in basal levels, usually ran concurrent with the decreased motility,

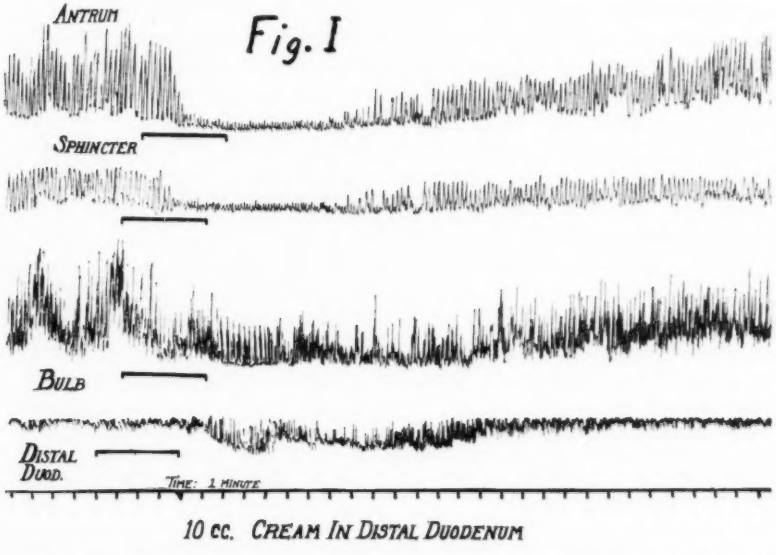

Fig. 1. Action of 10 cc. cream (30 per cent butter fat) introduced into the distal duodenum on the motor activity of the antrum, pyloric sphincter, duodenal bulb and distal duodenum. Balloon-water manometer registration. Time indicated in one minute intervals.

although the minimal tone level usually developed after 10 min. Loss of tone was usually moderate in degree in all four regions, but occasionally the loss was marked in the distal duodenum. Recovery of motility and tone of the entire sphincter region usually began in about 15 min. and was complete in 20 min. Administration of a second injection of 10 cc. of cream 30 min. following the first portion reproduced the previous series of events.

Greater quantities of cream up to 30 cc. prolonged the effect so that complete recovery required as long as 30 min. The effects on the antrum

and sphincter from large volumes of cream were not otherwise altered except that complete inhibition of the bulb and distal duodenum was of more frequent development. Small quantities of cream (2–4 cc.) produced complete inhibition of the antrum and sphincter persisting for 1 to 2 min. The influence on the bulb was much less marked and the distal duodenum was practically unaffected. In an attempt to simulate gastric evacuation of a fatty meal, a series of such small injections was made. They produced similar effects on each occasion and motility in the antrum and sphincter could be kept depressed for periods in excess of 90 min. Recovery occurred shortly after the administration of the last dose.

While recording pressures by the optical manometer-fluoroscopic technic from the antrum and bulb of fasting animals we administered 5 cc. of cream (15 expts.) into the duodenum 3 cm. distal to the sphincter. A

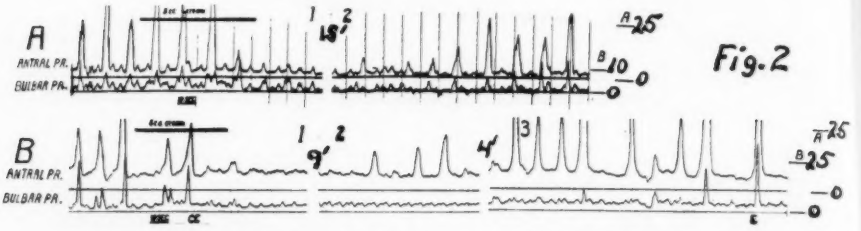

Fig. 2. Records of antral and bulbar pressures obtained by optical manometer technic before and after administering 5 cc. of cream into the proximal duodenum. Time intervals, 10 sec.

A. From a fasting animal. Between *1* and *2* a period of 15 minutes elapsed.

B. From an animal fed cornmeal mush. Period elapsed between *1* and *2* was 9 minutes. Four minutes elapsed between *2* and *3*. Gastric evacuation ceased at *CE*; evacuation returned at *E*.

decrease in the magnitude and frequency of the phasic pressure waves of the antrum and bulb began in about 50 sec. and progressed to complete cessation by 1.5 to 3 min. in the antrum and 3 to 4 min. in the bulb (fig. 2). Occasionally a transient stimulation of the bulb preceded the inhibition. During this excitation interval the frequency and magnitude of the bulbar pressure changes might be doubled and the basal pressure was moderately elevated. This stimulation apparently developed in an effort to empty the bulb and it was followed by a period of bulbar inhibition. Recovery was gradual and complete restoration of pressure waves occurred in the bulb after 8 to 10 min., but only after 13 to 15 min. in the antrum.

When the influence of fat had become maximal, the basal pressure had fallen more in the antrum (2 to 4 cm. water) than in the bulb (usually 0 to 2.5 cm. water). The antral-bulbar basal pressure gradient, normally 3 to 4 cm., fell to between 0.5 and minus 2.5 cm. Three to four cubic

centimeter doses of cream produced results in 12 experiments similar to those obtained with 5 cc., but inhibition of the sphincter region developed slightly later, was less complete and disappeared earlier. Frequently, no alteration in the behavior of the duodenal bulb could be detected with the 2 cc. quantities of cream, but a second administration of the same volume of cream 5 to 8 min. later almost invariably produced a transient inhibition.

Fed animals. Werle et al. (5) employed the fluoroscopic-optical manometer technic on fed animals and demonstrated that gastric evacuation was dependent on propulsive contractions of the distal antrum combined with a positive antral-bulbar basal pressure gradient (evacuation period A) and a positive antral-bulbar phasic pressure gradient (evacuation period B). We have demonstrated that fats in the duodenum of *fasting* animals inhibited the entire sphincter region and lowered the antral-bulbar basal and phasic pressure gradients. This induced us to extend the fluroscopic-optical manometer studies to fed animals in an attempt to explain on a quantitative basis the well established fact that fats retard gastric evacuation.

The influence of cream introduced into the duodenum on the behavior of the sphincter region was investigated in 36 experiments following the administration of food. Twenty-five grams of cornmeal was cooked for one hour; 80 grams $BaSO_4$ were incorporated and the mixture made up to a volume of 500 cc. This material was introduced into the stomach through the gastric cannula and an increase in basal pressures and in frequency and magnitude of the phasic pressure waves of the antrum and bulb was observed.

The food administered *via* the cannula was evacuated without a preliminary pause; frequently evacuation began during the feeding process. The antral and bulbar phasic pressure waves were of increased magnitude and occurred at a frequency of 5 to 6 per minute. The first 2 to 3 peristaltic antral waves gradually increased in maximal pressure. Subsequent waves appeared to divide the antrum into two distinct cavities and almost the entire mass distal to the wave was evacuated at each cycle. The volume evacuated by each cycle was dependent on the depth of the antral wave and the portion of the antrum at which it attained its maximum depth. We estimated that 2 to 4 cc. of gastric contents were discharged at each cycle. Typical basal pressures were: antral, 6 to 8 cm. of water; bulbar, 2 to 4 cm.; maximal phasic pressures were antrum 25 to 90 cm., bulb 20 to 75 cm. Usually these conditions persisted until the stomach was half emptied, then the antral and bulbar basal pressures, the frequency and magnitude of phasic pressure changes and the volume evacuated at each cycle gradually decreased slightly. Our observations are not in accord with the usual report of increasingly powerful contractions as the stomach empties.

After studying the evacuation process and the pressure changes for variable intervals, 5 cc. of cream (25 per cent B.F.) was introduced into the proximal duodenum in 11 experiments. Antral and bulbar motility and pressure waves were completely inhibited and gastric evacuation ceased (figs. 2, 3). Inhibition began in the antrum in 70 sec., in the bulb in 2 min., and became complete in the antrum in 2 to 3.5 min., and usually in the bulb in 3.5 to 4 min. Basal antral and bulbar pressures fell (antral more than bulbar), so the basal pressure gradient varied between plus 1 and minus 2, and regurgitation from the bulb into the antrum was occasionally observed. Reversed peristalsis in the sphincter region was not observed but "to and fro" movement of material between the bulb and the midportion of the duodenum was usually exaggerated.

Similar quantities of cream inhibited the sphincter region of fed animals less completely and for shorter intervals than in fasting animals. Complete recovery of the basal pressures, phasic pressure changes and peristaltic contractions occurred in the bulb after 7 to 9 min., and in the antrum about 4 min. later, but gastric evacuation did not begin to return until 15 to 20 min. after the beginning of cream administration. Cream was less effective in inhibiting the sphincter region of fed animals, partly because it was flushed out of the proximal intestine and diluted by the evacuated chyme and also because the presence of food in the stomach augmented the propulsive activity of the antrum.

An augmented contraction of the sphincter was not responsible for the retarded gastric evacuation following cream administration. Studies of the movements of shot sutured to the serosa at either side transversely of the sphincter or records obtained from a miniature balloon placed in the sphincter and arranged to record from an optical manometer showed the sphincter relaxed or, at the most, contracting rhythmically but feebly at this time. This observation is in accord with the results obtained in fasting animals by the tandem balloon technic.

Cream delayed gastric evacuation *chiefly* by depressing propulsive antral peristalsis and thus terminated the development of positive antral-bulbar phasic pressure gradients. The decrease or reversal of the basal antral-bulbar pressure gradient (evacuation phase A) and the increased bulbar activity which sometimes transiently occurred played minor rôles in retarding evacuation.

Recovery of antral contractions usually preceded the reëstablishment of gastric evacuation. The first antral contractions were not of the "propulsive type." Frequently they progressed at a slow rate, were shallow and tended to die out before reaching the sphincter. Such waves did not produce phasic pressure variations. Even when the contraction waves were normal in rate and depth and progressed to the sphincter they failed to occlude the lumen, for the antrum at this time was relaxed to approxi-

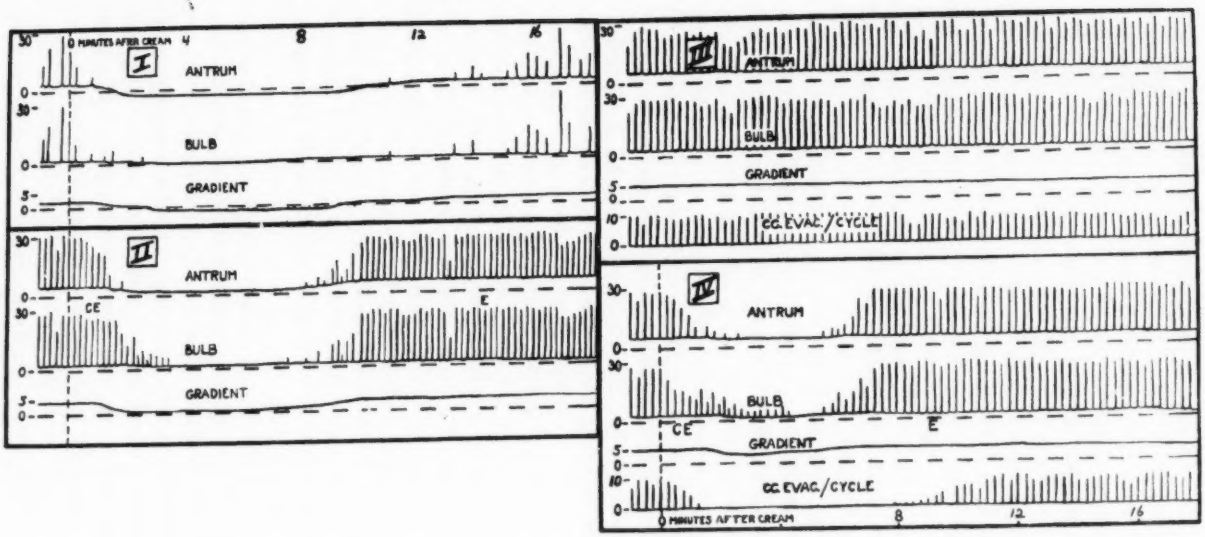

Fig. 3. Graphic representation of pressures obtained with the optical manometer technic from the pyloric antrum and duodenal bulb and the antral-bulbar basal pressure gradient before and after the injection of 5 cc. of cream into the duodenumat 0 minute. Pressures in centimeters water indicated by scale at left side of diagrams. *CE* signifies time of cessation of evacuation, *E* return of evacuation. I, animal fasting for 18 hours. II, animal fed cornmeal mush and $BaSO_4$. III, animal fed cornmeal mush and $BaSO_4$, duodenal drainage, no cream administered. IV, animal fed cornmeal mush and $BaSO_4$, duodenal drainage, 5 cc. cream injected into the duodenum.

mately twice the normal diameter. Thus, the waves produced slight antral phasic pressure changes and the antral contents were not discharged into the duodenum but returned to the body of the stomach. A similar situation was frequently observed during the period which normally elapsed between feeding and the beginning of gastric evacuation.

When 10 cc. quantities of cream were employed (15 expts.) the effects were similar to those obtained with 5 cc. doses, but were more striking and less variable. Complete inhibition of the bulb was sometimes absent with 5 cc. doses, but was always obtained with 10 cc. of cream. The decrease in basal pressure gradient was more constant and more marked; values of minus 2.5 cm. were reached. With small doses of cream (2 to 3 cc.) cessation of gastric evacuation was a more uniform effect than any one of the other phenomena mentioned.

Duodenal drainage. In ten experiments a rubber balloon was introduced into the duodenum about 10 cm. distal to the sphincter. By means of a catheter this balloon was inflated with air to the minimal pressure (20 to 25 cm. water), which largely but not absolutely prevented progress of duodenal contents beyond this point. A second catheter passed through this balloon to permit the introduction of cream into the duodenum about 17 cm. distal to the sphincter. The duodenal balloon and injection tube were inserted 30 min. preceding the experiment proper. A third tube was placed with its open end near the bottom of the duodenal cannula and suction applied so this tube provided duodenal drainage. The volume of duodenal contents collected was periodically measured.

This procedure was designed to study the effect of cream on gastric evacuation and the behavior of the sphincter region when dilution and flushing away of the cream by the chyme was prevented. The results, however, were complicated by the fact that duodenal drainage definitely hastened gastric evacuation. In our experiments the evacuation rate was increased 2 to 4 times. The experiments further differed from the other optical registration experiments since the cream was injected 15 cm. lower in the duodenum and exposure of cream to the action of bile and pancreatic juice was largely prevented.

In the experiments involving duodenal drainage, the fluoroscopic observations showed that the evacuated material passed directly through the bulb and proximal duodenum into the collecting bottle. Frequently the antral contraction forced the chyme without pause as far as the duodenal cannula and contraction of the proximal duodenum was not required for the propulsion. Thus the volume evacuated by each antral peristaltic wave could be measured. After feeding, the first few cycles which evacuated gastric contents discharged small volumes. For the next 3 to 7 min. the volume per cycle was rather irregular but averaged about 10 cc. (occasionally increased to 50 cc.), then gradually declined to a steady state in which

6 to 8 cc. were discharged per cycle until the stomach was almost completely emptied.

Apparently the rate of gastric evacuation normally represents a balance between *a*, an augmenting effect produced by the distension of the antrum and body of the stomach by food; *b* an inhibitory influence produced by the presence of the evacuated material in the intestine, acting in part through mechanical (nonspecific) influences and partly through chemical (specific) factors. Elimination of factor *b* in these experiments releases the evacuating power of the stomach from an important fraction of the intestinal regulation.

Introduction of 5 cc. of cream into the duodenum of fed animals provided with duodenal drainage produced results similar to those obtained from similar doses without duodenal drainage (fig. 3). However, the effects were slower in development, the suppression of phasic pressure waves (especially of the bulb) and gastric evacuation was less complete and of shorter duration in the animals with drainage. As in the experiments without duodenal drainage, the first antral peristaltic waves to return after cream inhibition failed to expel the antral contents into the intestine. Instead, this material usually returned to the body of the stomach for the peristaltic waves did not sufficiently occlude the antral lumen to prevent a retrograde axial stream. Four cubic centimeters was the minimal quantity of cream which was even transiently effective in the drainage experiments.

Gastric distention by a balloon. Stretching the stomach may *per se* produce some of the effects noted from feeding. We distended the body of the stomach of fasting animals with a large balloon and again determined the modification in the activity of the sphincter region and the effectiveness of fat administration. No significant alteration in the activity of the pyloric region was noted with 100 to 150 cc. of air in the ballon. When the distending volume was 500 cc. of air, the activity of the pyloric region approached but did not equal that obtained by administering *via* the cannula 300 cc. of food. Thus, distention of the body of the stomach is one of the factors responsible for the difference in sphincter region activity characteristic of the fed and fasting states. Food stimulates the sphincter region more than a balloon distended to an equal volume. This may be due to the fact that the food, unlike the balloon, enters the sphincter region. Confirming this is the observation that the tandem balloons in the sphincter region gave results from fasting dogs which resembled those obtained by optical registration from fed rather than fasting animals. Apparently, the moderate distention of the sphincter region augmented the motility in that region.

In four experiments with the body of the stomach of fasting animals distended by 500 cc. of air, the administration of 5 cc. of cream into the

proximal duodenum inhibited the sphincter region in a manner similar to the characteristic response observed in the fasting animal whose stomach was not distended. However, the cream inhibition was slightly less marked in the animal with a distended stomach; the period of complete antral inhibition, for example, being 1 to 2 min. shorter.

SUMMARY

In studies made with the tandem balloon method the introduction of fats into the duodenum of fasting dogs inhibited the motility of the pyloric antrum, sphincter and duodenal bulb. A quantitative study of the pressure relations in the pyloric sphincter region combined with a visualization of motility and propulsion was obtained by the optical manometer-fluoroscopic technic. Employing this method, it was again shown that cream in the duodenum inhibited the pyloric region and further proved that cream decreased or reversed the antral-bulbar basal and phasic pressure gradients. The action of cream was most pronounced in fasting animals, then in order of decreasing effectiveness, fasting animals with the stomach distended by a large balloon, fed animals, fed animals with duodenal drainage. Fats retarded gastric evacuation chiefly by decreasing antral propulsive peristalsis. Sphincter spasm was not involved; on the contrary, evacuation was retarded in spite of sphincteric relaxation. The rate of gastric evacuation represents a balance between *a*, the augmenting effect produced by a distention of the antrum and body of the stomach by food, and *b*, the inhibitory influence produced by the presence of chyme in the intestine, acting in part through mechanical (nonspecific) influences and partly through chemical (specific) factors. In experiments involving duodenal drainage, factor *b* was largely eliminated, thus the evacuating mechanism of the stomach was released from an important fraction of the intestinal regulation and the evacuation rate was augmented.

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THE INFLUENCE OF ESTRADIOL ON THE SECRETION OF GONADOTROPIC HORMONE IN ADULT PARABIOTIC RATS[1]

ROLAND K. MEYER AND CLYDE BIDDULPH

From the Department of Zoology, University of Wisconsin, Madison

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We have previously reported that the ovaries of a normal immature female parabiotic rat undergo hypertrophy following gonadectomy of its partner, and that the daily injection of relatively small quantities of estrogen and somewhat larger quantities of androgen into the castrated partner of such pairs prevents ovarian enlargement in the normal animal (1–5). These results were interpreted to mean that the estrogen or androgen prevented the hypersecretion of gonadotropic hormone from the pituitary gland of the castrated rat which occurred following gonadectomy.

Using adult parabiotic rats, Hill (6, 7) and Witschi and Levine (8) demonstrated that the pituitary gland of the partner which was gonadectomized underwent hypersecretion, for the normal female partner developed a condition of continuous estrus. Because it was possible to prevent hypersecretion of gonadotropic hormone in immature pairs by the injection of estrogen, it became of interest to determine whether estradiol[2] acted similarly in adult pairs which had developed a continuous vaginal estrus.

MATERIALS AND METHODS. Female littermate rats weighing 70 grams or more were united in parabiosis at 31 to 33 days of age according to the method of Bunster and Meyer (9), except that metal skin clips were used instead of silk sutures in closing the skin incisions. Ether anesthesia was used and sterile precautions were taken during the operation. The right-hand partner was ovariectomized at the time of parabiotic union. Following the opening of the vagina of the normal partner, which usually occurred 7 to 8 days following parabiotic union, the vaginal smear was followed to determine the time at which continuous estrus appeared. After a period of 25 to 30 days of continuous estrus, injections of 1 γ of estradiol per day were made into the ovariectomized partner. The amount of

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[2] We are indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey, for the estradiol used in the experiments.

hormone injected each day was contained in 0.05 cc. of corn oil. At varying intervals the dosage of estradiol was increased to 2, 3 or 4 γ per day in some of the pairs, as is indicated in the figure. The ovaries of the normal partner were examined under a binocular dissecting microscope during laparotomies which were performed before or during the estrogen treatment. In pairs 3, 5 and 7 one ovary was removed for histological study before the estradiol injections were begun. In pairs 5 and 6 the ovariectomized partner was hypophysectomized after the normal partner had shown a second extended period of continuous estrus following withdrawal of the initial estrogen treatment.

Records of representative pairs are presented in figure 1. It should be emphasized that the figure presents the vaginal smear record of the normal female partner, and that the injections of estradiol were always made into the ovariectomized partner. The notations concerning estrogen treatment of the pairs refer to the time at which injections were either begun or discontinued. Day 0 is the first day of continuous estrus, and all successive days are dated from this time. Laparotomies of the normal partner are indicated by the letter *L*. The injections of the luteinizing hormone, *LH*, were made into the ovariectomized partner of pair 7.

Results and discussion. The data were obtained from 14 pairs of rats, and of these three were treated a second time with estradiol, so the total number of experiments was 17. Records of 7 pairs are included in the figure.

The vaginal smear records show that following injections of estradiol into the ovariectomized partner, the continuous estrus of the normal partner was replaced by a temporary diestrus. Usually 1 γ of estradiol per day was sufficient to cause the vaginal change, although the dosage was raised to 2 γ per day in one pair before this change occurred. Laparotomies performed before estrogen injections were made showed that the ovaries were composed of numerous large follicles, some of which were cystic. These observations were confirmed by histological sections of ovaries removed at this time.

The diestrus which followed the estradiol injections varied from 1 to 20 days, the average duration being 10 days. The ovaries of the normal partner of all the pairs examined except one (not included in figure), contained follicles which now appeared to be smaller than those present at the beginning of the estrogen treatment. The exceptional pair had three corpora lutea in one ovary. The diestrus of the normal female was followed by estrous cycles, which were often somewhat irregular in some of the pairs. However, by increasing the dosage of estradiol to 2 γ per day, they tended to become more regular (pairs 2, 3 and 5). It was necessary to increase the dosage to 3 γ per day in one pair before regular cycles were obtained. The estrous cycles of the normal female persisted as long as the ovariectomized rat was injected with estradiol. Laparotomy of the normal

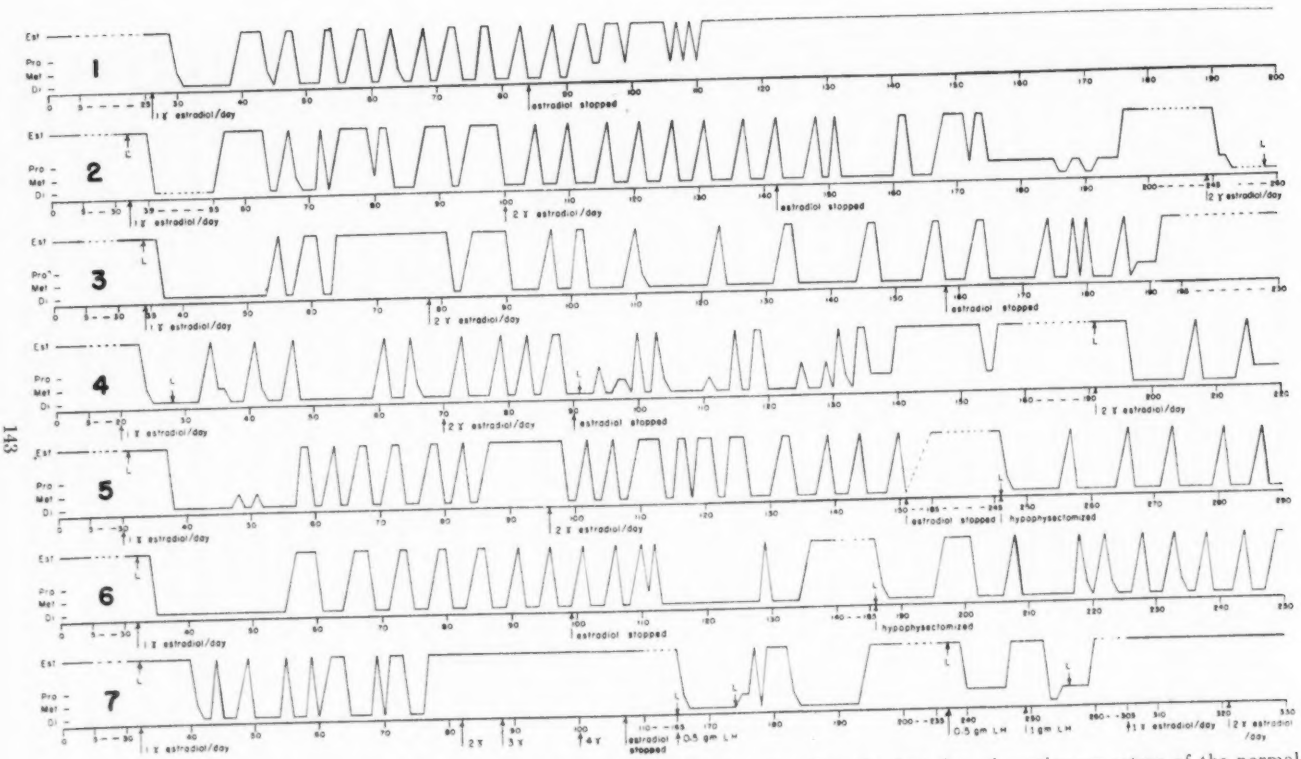

Fig. 1. The vaginal smear record of the normal partner is represented. Day O is the first day of continuous estrus of the normal parabiont following ovariectomy of the other. The ovariectomized partner received the injections. The ovariectomized rat of pairs 5 and 6 was hypophysectomized. Laparotomy of the normal partner is represented by the letter *L*.

partner of pair 4 during this period showed that the ovaries were composed of follicles and corpora lutea. Following cessation of the estrogen treatment, several normal estrous cycles usually occurred, after which the cycles became irregular and finally continuous estrus reappeared.

The ovariectomized partner of pairs 5 and 6 was hypophysectomized after an extended period of continuous estrus which followed the withdrawal of the initial estradiol treatment. Here, as during the estradiol injections, the continuous estrus of the normal female was replaced by a diestrus, after which normal cycles appeared. Pair 5 had 16 regular cycles, and pair 6 had 17 after hypophysectomy and before autopsy.

Comparison of the results following the injection of estradiol with those found after hypophysectomy suggests that the estradiol prevented the secretion of gonadotropic hormone from the pituitary gland of the ovariectomized rat. Moreover, the administration of the proper amount of estradiol was apparently as effective in bringing about regular estrous cycles in the normal partner as was the removal of the pituitary gland. Pair 7 appears to be an exception to this since two of the three periods of estradiol treatment were not effective in changing the continuous estrus to diestrus. During the first treatment, a short period of continuous estrus appeared while 1 γ of estradiol per day was being injected. Consequently the dosage of estradiol was increased to 2, 3 and finally 4 γ per day, but these amounts were ineffective in changing the vaginal smear. Also, at a subsequent time, injections of 1 and 2 γ of estradiol per day were made, without changing the type of vaginal smear. From these results it appears that the pituitary gland of the ovariectomized rat of this particular pair became refractory to the estrogen treatment.

Injections of LH were made into the ovariectomized partner of pair 7 to determine whether this substance could cross to the normal parabiont and luteinize the follicles. After each of 3 injections of LH, the vaginal smear of the normal partner changed from continuous estrus to diestrus and corpora lutea were found in the ovaries (see figure). It will be noted that the diestrus which occurred after each injection was short and was followed by periods of prolonged estrus. The results of this experiment demonstrate that the ovaries of the rat in continuous estrus are sensitive to LH and that LH can be transferred from one parabiont to the other. This conclusion is of importance in explaining the cause of the period of prolonged diestrus which followed immediately after the injection of estrogen into the ovariectomized partner. In all the pairs examined during this period except one, the ovaries were found to be small and devoid of corpora lutea. These results and those obtained by injecting LH into the ovariectomized partner of pair 7, provide evidence for the concept that the injection of estrogen in the amounts used in these experiments, does not bring about the release of sufficient quantities of LH from the pituitary gland of the ovariectomized partner to luteinize the ovaries of the normal

parabiont. Hohlweg (10) and Fevold, Hisaw and Greep (11), however, have shown that estrogen causes the formation of corpora lutea in single normal immature and mature rats. This luteinization of the ovaries was attributed to a release of LH from the pituitary gland by the injected estrogen.

It seems that the diestrus of the normal rat, which followed the injections of estradiol into the ovariectomized parabiont, can be explained best on the basis that the injections of estradiol prevented the secretion of gonadotropic hormone from the pituitary gland of the ovariectomized rat. It is also suggested that the length of the diestrus represents the time required for the pituitary gland of the normal partner to recover from the inhibiting influence of the estrogen produced by its own ovaries during the time that they were continuously stimulated by the gonadotropic hormone from the castrated partner. Therefore, it would seem that the appearance of estrous cycles in the normal animal after the period of prolonged diestrus represents the resumption of cyclic activity by the pituitary gland and ovaries of this rat.

Evidence that there was very little, if any, transfer of the injected estrogen from the ovariectomized partner to the normal, is furnished by the occurrence of normal estrous cycles in the latter during the period of injection. If the injected estrogen were present, it would no doubt have interfered with the estrous cycles of the normal partner, for it was determined in a group of normal female rats that the injection of 1 γ of estradiol per day was sufficient to cause continuous vaginal estrus. Therefore, it seems unlikely that the estrogen passed from the injected to the normal partner in amounts sufficient to interfere with the normal cyclic activity of the pituitary gland and ovaries.

One pair, which is not included in the figure, was not in continuous estrus when injections of estradiol were begun, and the subsequent cycles were very irregular and appeared to be no different from those obtained before beginning the injections. Laparotomy 29 days following the beginning of injections showed that there were many corpora lutea in the ovaries of the normal partner. Subsequent treatment with 2 and 3 γ per day likewise did not produce regular cycles. Furthermore, the normal partner did not remain in continuous estrus after the injections of estradiol were stopped.

Hill (6) reported that usually 3 months' time was required following ovariectomy before continuous estrus was attained. Our rats required considerably less time to reach this stage, since the average time was 39 days. Kawashima also noted a shorter period of time than that required by the rats used by Hill (cited by Hill).

In immature female-female pairs, it was found that 0.025 γ of estradiol per day was required to prevent hypersecretion of the gonadotropic complex following ovariectomy (4). In mature pairs, from 1 to 3 γ of estradiol

per day have been found to be necessary. However, this difference in the two studies should be pointed out, namely, that the estrogen treatment was begun immediately following gonadectomy in the immature pairs, whereas it was begun after the hypersecretion had become well established in the adult pairs. In the former case there was probably very little gonadotropic hormone being secreted by the pituitary gland, whereas in the latter, gonadotropic hormone secretion was at a very high level. Although no attempt was made to determine the minimum amount of estradiol required to prevent castration hypersecretion in the adult pairs, it would seem that they probably require more estrogen to prevent hypersecretion of the gonadotropic complex than do immature parabiotic rats.

SUMMARY AND CONCLUSIONS

Continuous vaginal estrus was obtained in the normal partner of female parabiotic rats at an average of 39 days following ovariectomy of the other partner.

After an extended period of continuous estrus, injections of estradiol were made into the ovariectomized rat. As a result of these injections, the continuous vaginal estrus of the normal partner was replaced by a diestrus, which persisted an average of 10 days. Normal estrous cycles then appeared and usually persisted as long as the injections of estradiol were continued. After stopping the injections, the estrous cycles became irregular and finally continuous estrus reappeared. This treatment was repeated several times in some of the pairs with the same results.

The ovariectomized partner of pairs in continuous estrus was hypophysectomized, and, as during the estradiol treatment, the continuous estrus of the normal partner was replaced by a diestrus. Normal cycles then appeared and persisted until the rats were autopsied.

Because the results following the injection of estradiol into the ovariectomized partner were similar to those following hypophysectomy, it is concluded that estrogen prevents the hypersecretion of gonadotropic hormone by the pituitary gland of adult ovariectomized rats.

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THE MECHANISM OF DEFLATION HYPEREMIA IN THE INTESTINE

HAMPDEN LAWSON

From the Department of Physiology, University of Louisville School of Medicine

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The most consistent, and one of the most striking, effects of low-pressure distention of the dog's small intestine (below 40 mm. Hg lumen pressure) is the period of increased arterial flow beginning simultaneously with the deflation and lasting 20 to 30 seconds or longer. This period has been described as the third phase of the total response (Lawson and Chumley, 1940). In the present report it will be called deflation hyperemia. As a prominent spike of augmented flow it either terminates the response or passes more or less abruptly into the terminal fourth and fifth phases, with which this report is not concerned.

Deflation hyperemia is not materially affected by mesenteric denervation of the loop, yet is abolished or markedly reduced by local treatment of the loop with cocaine. It is not seen following low-pressure inflations if the loop is encased in plaster to prevent enlargement. On the basis of these and similar data it has been suggested that it represents the persistence for a short time beyond the distention period of vasodilatation (or an extravascular resistance-lowering mechanism) set up through the peripheral nervous apparatus by stretch of the gut walls. This interpretation of deflation hyperemia, although compatible with all the data available, cannot be regarded as final without further study. Treating the loop with cocaine or encasing it in plaster would not only abolish hyperemia from the mechanism postulated, but might also, through an alteration in the mechanics of the loop, modify all phases of a response which is wholly mechanical, or primarily mechanical, with secondary non-nervous phases.

The present report considers two non-nervous mechanisms which might account for an increased arterial flow into the intestine immediately following distention, in an attempt to evaluate their contribution to deflation hyperemia: 1, the rapid filling under a steepened pressure gradient of vessels the volume of whose contents has been diminished during the distention; 2, post-ischemic vasodilatation (reactive hyperemia) in portions of the intestine deprived of blood by the distention.

The first mechanism, a filling phenomenon, has been held responsible by Rössler and Pascual (1932) for an overshoot in coronary flow which they

recorded at the beginning of diastole, and by Anrep, Blalock and Samaan (1934) for a similar phenomenon observed in skeletal muscle at the termination of a tetanus. As described for these tissues it is of very brief duration (less than 0.2 sec. for skeletal muscle). Its duration in the intestine during deflation would depend upon the rate of inflow and the blood volume to be replenished as well as upon the rate of withdrawal of the compressing force. In the absence of data on these values it is unwise to conclude that this factor could not account for deflation hyperemia lasting usually 20 to 30 seconds, and sometimes longer than 1 minute.

The fact that deflation hyperemia bears no relationship to the gross flow deficit incurred during the distention, and is observed even when there has been an increase in flow (Lawson and Chumley, 1940b), does not exclude the mechanism of reactive hyperemia, since redistribution of blood in the distended loop may leave some regions ischemic, the local ischemia being masked by an increased flow in other regions. In fact, unless the resistance-lowering mechanism which is set up by stretch acts to maintain the flow through the compressed regions, masked ischemia would be expected to develop in these regions during the distention, to cause reactive hyperemia on deflation.

A third possibility, that the overshooting on deflation is due to properties of the flow-meter (Lawson, 1940a), has not merited separate treatment, since no overshooting is recorded when arteries drained through cannulas are clamped or released or when the flow-meter installation is tested with suddenly changed flows in artificial systems, and since deflation hyperemia with identical features has been recorded with other types of differential manometers (independent mercury manometers—see fig. 1; inverted U-manometers—see Lawson and Chumley, 1940).

METHODS AND RESULTS. Short loops of ileum were prepared in barbitalized dogs as already described. The distentions employed were similar in duration (usually less than 2 min.) and magnitude (usually less than 40 mm. Hg distending pressure) to those of the previous study. In most of the data presented here the metal bellows manometer (Lawson, 1940) was used to read the pressure fall across the applied constriction in the mesenteric artery. Recorded pressure differences were converted into volume flow of blood by calibration *in situ* at the close of each experiment. The pressure difference, plotted as zero, for zero flow into the loop was obtained by clamping the artery in the mesenteric pedicle of the loop (below the constriction and the lower cannula). With this clamp in place to stop flow into the loop, a second artery below the constriction or a side arm on the distal cannula was opened and flow through this outlet regulated with a screw clamp to produce manometer deflections within the limits observed in the experiment. Three to five such deflections were plotted against their corresponding measured flows to obtain a calibration curve

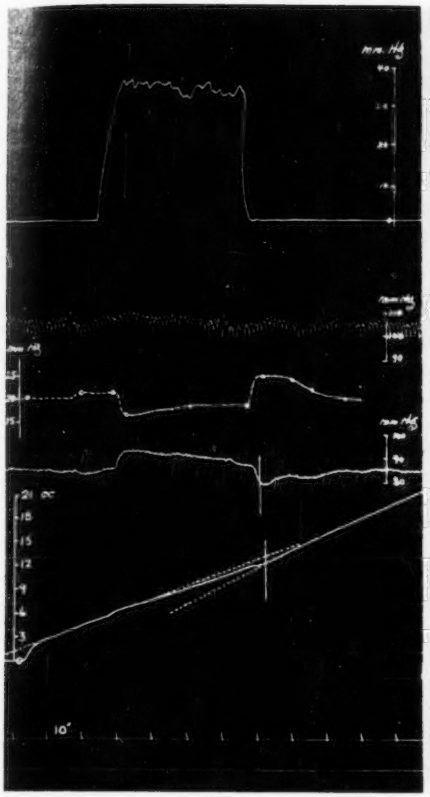

Fig. 1

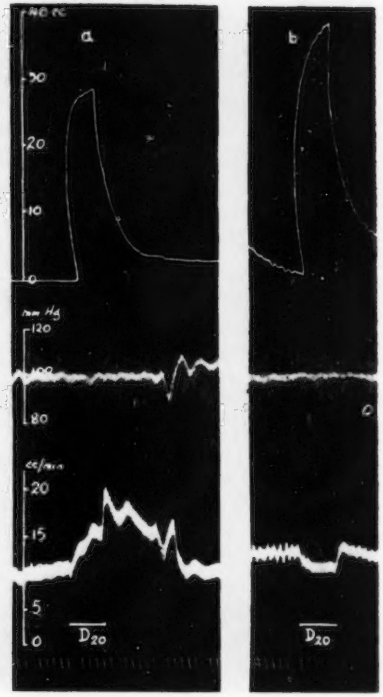

Fig. 2

Fig. 1. The uppermost record is a tracing of distention pressure. The second is a tracing of carotid pressure, the fourth a tracing of mesenteric arterial pressure below the constricting clamp, recorded by independent mercury manometers. A qualitative flow record comparable to those in the other figures is constructed by plotting (third tracing) the pressure difference at the points marked by circles, between the two manometers. The lowermost record is a tracing of venous flow, made by recording air displacement from a flask receiving the outflow from the cut vein. The upper dotted line drawn on the outflow record is a continuation of the control rate of flow, the lower dotted line a continuation of the increased rate of flow following deflation. Corrected simultaneous ordinates are drawn through the venous outflow record at the point where rate of flow begins to exceed the control, and through the record of mesenteric arterial pressure. Time in 10-second intervals.

Fig. 2. Uppermost tracing records the volume of fluid accepted by the loop from the distention reservoir. The second tracing is a record of carotid pressure, the lowermost a record of arterial flow into loop (mesenteric denervation, weight 34 grams). The signals mark distention at a pressure of 20 cm. water. Time in 10-second and 1-minute intervals. *a* shows the response before, and *b* after 10 cc. 1 per cent cocaine hydrochloride had been substituted for an equal volume of loop fluid. (Loop distended with 0.9 per cent NaCl solution directly from reservoir, without balloon.)

for each experiment. The plotted data sometimes lay on a straight line, but usually on a smooth curve convex toward the flow axis. Extrapolation on the calibration curve to the true zero pressure axis usually showed a flow past the constriction of 0.5 to 2.0 cc. per minute with the loop clamped off. This small uncalibrated flow never amounted to more than 10 per cent. of the total flow past the constriction, usually considerably less. It goes to the pedicle central to the occluding clamp, and to sessile lymph nodes along the axis of the superior mesenteric artery peripheral to the constriction. That it remains fairly steady through procedures such as are employed in the present study is suggested by the relatively fixed level of residual flow recorded when flow to the loop is stopped (fig. 4).

Expressed as volume flow per minute per 100 grams of postmortem intestinal weight, the average control flow into six innervated loops was 36 cc., with extremes of 25 and 48 cc.; into eight loops with mesenteric denervation, 42 cc., with extremes of 30 and 55 cc. These values agree reasonably well with the value of 31 cc. per 100 grams per minute obtained by Burton-Opitz (1908) for venous flow from innervated small intestine under similar experimental conditions.

The rôle of the filling mechanism. That time is required for the suddenly decompressed vascular bed to fill up was suggested by Burton-Opitz (1908) in explanation of a brief stoppage of venous flow from the intestine during deflation. The stoppage of outflow during the first portion of deflation has been repeatedly confirmed in the present investigation (Lawson and Chumley, 1940). If the increased inflow which begins at the same time is due altogether to low pressure in the decompressed vascular segments, it should terminate as soon as pressure in these segments has returned to normal. Other things being equal, changes in venous outflow from the loop may be taken as an index to pressure changes in the segments which are being compressed and decompressed by distention and subsequent deflation. Under these conditions it may be concluded that pressure in peripheral segments during decompression has returned to its control value as soon as venous flow recovers its control value.

In a total of twelve satisfactory observations on four dogs, venous outflow recovered or exceeded its control value within 2 to 8 seconds after the beginning of deflation. Simultaneous records of inflow and outflow usually showed that the peak of deflation hyperemia was reached later than this (fig. 1). From 75 to approximately 95 per cent of the total excess flow (area between control and hyperemic flow levels) always occurred more than 10 seconds after the beginning of deflation, at a time when venous flow, on the basis of these data, should have recovered or exceeded its control value. During the greater part of the period of deflation hyperemia both inflow and outflow are increased.

If filling of the decompressed vascular segments is solely responsible for

deflation hyperemia, the excess volume of flow on deflation should equal the volume reduction in the vascular bed during distention. An attempt to obtain data on the reduction in vascular volume in the distended loop by gravimetric oncometry (Lawson, 1940b) gave equivocal results. The loop sometimes increased, and sometimes decreased in weight, the changes usually being small. Data obtained by more conventional methods are presented in table 1. The volume increase in the lumen of the loop (ΔV_i) was recorded by the method already described (Lawson and Chumley,

TABLE 1

For explanation see text

DOG NUMBER	DISTENDING PRESSURE	$\Delta V_e - \Delta V_i$ (cc.)			DEFLATION HYPEREMIA
		10"	20"	Terminal	
	cm. aq.				*cc.*
1	20	+0.2	±0.0	+0.5	0.05
	30	−0.5	−0.6	−0.7	0.33
	30	−0.3	−0.9	±0.0	0.20
	30	+0.4	+0.8	+0.8	0.25
	40	−0.2	−0.5	−0.9	1.50
	40	±0.0	−0.9	−0.9	1.00
2	20	−0.2		−0.2	0.50
	30	+0.6		−0.4	0.35
	30	−0.2		−0.7	0.80
	30	−0.2		−1.2	0.50
	40	−1.2		−2.8	0.60
	50	−0.1		−2.1	0.70
	60	+0.1		−2.1	0.90
	70	−0.6		−3.2	1.90
	70	−1.1		−3.9	2.40
	80	−0.4		−3.9	3.30

Note: In dog 1 $\Delta V_e - \Delta V_i$ persisted as a positive value (increase in wall volume) and in dog 2 as a negative value (decrease in wall volume) for 1 to 3 minutes after deflation.

1940b). The external volume increase of the loop (ΔV_e) was determined simultaneously by placing the loop in the usual type of oncometry chamber, connected with a volume recorder. The value of $\Delta V_e - \Delta V_i$ represents the change in the volume delimited by the distending balloon in the interior of the loop and the peritoneal covering of the loop and pedicle on the outside. The erratic values which are shown in the table, in agreement with those obtained by the gravimetric method, suggest that extravascular fluids, either in the lumen or in the gut wall, may change their volume unpredictably during distention to mask the change in vascular volume. The initial volume change, read at the end of 10 seconds following infla-

tion, is probably more nearly a pure vascular volume change since the extravascular changes would be expected to be somewhat slower. The peak of inflow reduction has usually been passed at the end of 10 seconds' distention, and the enlargement of the loop is almost complete. The emptying of the vascular bed under compression is probably therefore complete. Examination of the table shows that in only three out of a total of sixteen distentions was the volume change at this time equal to more than 50 per cent of the excess volume flow during deflation; in five cases the volume change at this time was in the wrong direction and therefor could account for none of the deflation hyperemia, and in the remaining eight distentions the volume change could account for only 12 to 44 per cent of the hyperemia.

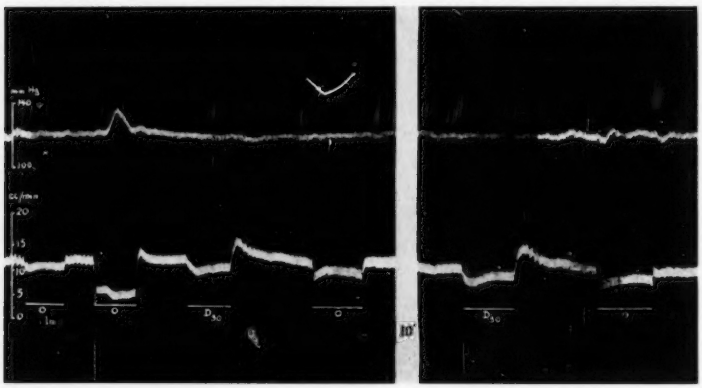

Fig. 3. Upper record is carotid pressure (mercury manometer), lower record arterial flow into loop (weight 30 grams). During the signals marked *O* the artery supplying the loop was partially occluded. During the signals marked *D* the loop was distended at a pressure of 30 cm. water. Timer marks intervals of 10 seconds and 1 minute.

The rôle of reactive hyperemia. It was assumed for the purposes of the present study that the post-ischemic behavior of the loop as a whole following a period of arterial occlusion can be taken as a model of the behavior of any tissue in the loop which might be subjected to ischemia during distention. Complete arterial occlusions were produced with a bull-dog artery clamp, or with a modified Goldblatt clamp, and partial occlusions with the latter, applied in the mesenteric pedicle of the loop. The artery was stripped of gross nerve fibres at the site of the clamp.

When the flow reduction produced by a distention was duplicated by partial arterial occlusion, the hyperemia following the distention was always the greater (fig. 3). The gradual increase in flow during partial occlusion

shown in the figure is typical of the partial, but not of the complete occlusions (see fig. 6). It resembles the gradual decrease in the pressure difference which is observed for 3 to 5 minutes after application of this type of clamp as the flow-meter constriction, and probably represents loss of tone in the segment of artery within the clamp. In most animals flow had to be reduced 30 to 50 per cent by arterial occlusion in order to elicit hyperemia. Gross flow reductions of this magnitude were never obtained

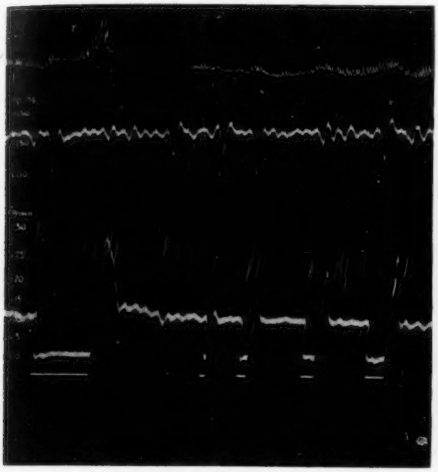

Fig. 4

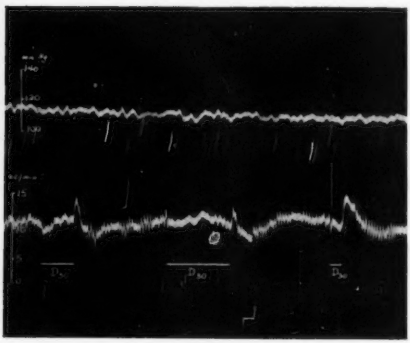

Fig. 5

Fig. 4. Uppermost tracing is a record of loop motility. Second tracing is a record of carotid pressure. Third tracing is arterial flow into loop (mesenteric denervation, weight 35 grams). The signals mark complete stoppage of flow to the loop by application of a bull-dog clamp to its artery. Time in intervals of 10 seconds and 1 minute.

Fig. 5. Upper tracing is a record of carotid pressure. Lower record is arterial flow into loop (mesenteric denervation, weight 27 grams). The signals mark loop distentions at a pressure of 30 cm. water. Timer marks intervals of 10 seconds and 1 minute. The small regular waves in flow at a rhythm of 8 to 10 per minute which appear in this record are usually synchronous with recorded rhythmic intestinal movements.

with the low-pressure distentions of this and the previous report. If all tissues in the loop share equally the reduced flow during partial arterial occlusion, the threshold flow reduction for all tissues, for the production of reactive hyperemia, must lie within or above this range. If, then, deflation hyperemia is merely reactive hyperemia in portions of the loop which have suffered supraliminal flow reduction during the distention, the flow through these portions must have been reduced at least 30 to 50 per cent by the distention.

Complete arterial occlusions of shorter duration than 20 seconds usually were followed by no increased flow. Prolonging the duration of the occlusion beyond this up to 2 minutes or longer increased the height and the area of the resulting hyperemia (maximum rate of flow and total excess flow) (fig. 4). In contrast, neither the height nor the area of deflation hyperemia increased with the duration of distention, provided the distention was sufficiently prolonged to permit fairly complete enlargement of the loop (fig. 5). In four dogs in which a study of this point was made, distentions prolonged to 2 minutes or longer were followed by smaller deflation hyperemias than distentions lasting 20 to 30 seconds.

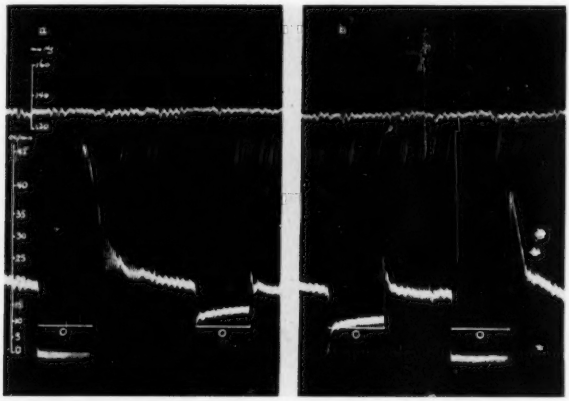

Fig. 6. Upper tracing is a record of carotid pressure. Lower record is arterial flow into loop (mesenteric denervation, weight 35 grams). The signals mark partial or complete stoppage of flow into loop with Goldblatt clamp applied to its artery. Time in 10-second intervals. *a* shows reactive hyperemia before, and *b*, after introduction of 10 cc. 1 per cent cocaine hydrochloride into lumen of loop. The flow deficits before and after cocaine are nearly equal.

The practically complete abolition of deflation hyperemia by local application of cocaine is shown in figure 2. With low-pressure distentions which cause an increase in flow over the control during the distention period, cocaine abolishes both the hyperemia during distention and the deflation hyperemia, as shown in the figure. In comparison, reactive hyperemia following arterial occlusion was relatively little affected by cocaine (fig. 6). As might be expected from any vasoconstrictor agent, the absolute values of reactive hyperemia in terms of the maximum rate of flow and the volume of flow excess were usually somewhat reduced by cocaine. In two animals an attempt was made to obtain a quantitative comparison of the effect of cocaine on the two types of hyperemia, by expressing all responses as percentage changes from the control rates of flow.

In one animal cocaine reduced deflation hyperemia 85 per cent, reactive hyperemia 57 per cent; in the other cocaine reduced deflation hyperemia 50 per cent, and increased reactive hyperemia 45 per cent (all figures are based on the average of 2 to 6 trials before and after cocaine).

Discussion. The reduction in vascular volume during distention should equal the excess volume flow on deflation only if no change other than compression and decompression occurs in the vascular bed. If the data afford even a rough approximation (and not even this can be claimed with certainty for them) of the amount of blood driven out of the peripheral segments during distention, it is clear that deflation hyperemia usually accomplishes much more than just the return of this volume. Insofar as the data are defensible, they show that the "filling" mechanism could account usually for no more than 45 per cent of the deflation hyperemia. Whether, as the data suggest, vascular volume may increase during distention, will have to be determined with more suitable methods.

The less questionable data on the time required for the decompressed vascular bed to fill up to its normal pressure levels and so restore venous flow suggest that no more than 25 per cent, and usually considerably less, of the deflation hyperemia could be due to this mechanism. This is probably a more accurate evaluation.

On the basis of these data it is probably justifiable to conclude that 75 per cent or more of the excess flow during deflation is the result of some other mechanism. That the other mechanism, which is largely responsible, is not reactive hyperemia, is suggested by its abolition with cocaine, and by its failure to increase with increasing duration of the inciting cause. It seems clear that in the distended cocainized loop the reduction in flow is not sufficient, in any tissue, to cause reactive hyperemia. Data to be published elsewhere show that the hyperemia following excessive distentions under pressures approaching arterial pressure, is not abolished or as greatly reduced by cocaine. It is likely that reactive hyperemia contributes to the excess flow on deflation only after such excessive distentions. The decrease of deflation hyperemia with increasing duration of the distention requires further study before an explanation can be offered.

These data, by excluding the two mechanisms studied from significant participation, support the interpretation of deflation hyperemia offered in the earlier report.

SUMMARY AND CONCLUSIONS

The increased arterial flow into loops of small intestine following periods of low-pressure distention (deflation hyperemia) is associated except for the first few seconds following deflation with an augmented venous flow, and usually is considerably greater in volume than the demonstrable volume

reduction of the gut walls during the distention. It is concluded that only a small portion (less than 25 per cent) of the deflation hyperemia can be due to the filling of vascular segments whose pressure is temporarily lowered during the period of decompression. The remainder of the deflation hyperemia differs strikingly from reactive hyperemia in its sensitivity to cocaine, and in its relation to the duration of the inciting cause. Its mechanism is therefore probably not identical with that of reactive hyperemia.

The assignment of a minor rôle to these two mechanisms makes it probable that the greater part of the deflation hyperemia is due to persistence of a resistance-lowering mechanism set up by stretch of the intestinal walls.

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PHENYLTHIOCARBAMIDE TASTE THRESHOLDS OF RATS AND HUMAN BEINGS

CURT P. RICHTER AND KATHRYN H. CLISBY

From the Psychobiological Laboratory, Phipps Psychiatric Clinic, Johns Hopkins Hospital

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Previous papers contain a description of a method for the determination of taste thresholds which can be used both for rats and human beings. With this method the taste thresholds have already been determined for the two most common substances which appear in our diet in purified form, common salt and sugar. Rats showed that they could first distinguish a sodium chloride solution from distilled water when the concentration reached 0.055 gram per cent (1); human beings first recognized a difference between sodium chloride solution and distilled water in concentrations of 0.016 per cent and first recognized a "salt" taste in concentrations of 0.087 per cent (2). Rats first showed that they could distinguish a sucrose solution from distilled water in concentrations of 0.5 per cent; human beings first recognized a difference between sucrose solution and distilled water in concentrations of 0.17 per cent and first recognized a "sweet" taste in concentrations of 0.41 per cent (3). Thus, for these two common substances human beings and rats have very nearly the same taste thresholds.

A further study has now been undertaken on the taste thresholds of a highly bitter tasting substance, phenylthiocarbamide. This substance was used partly because it has already been employed in numerous studies on taste thresholds (4, 5) and on the inheritance of taste ability (6, 7, 8, 9, 10, 11) and partly because, unlike sodium chloride and sucrose, it has a highly toxic effect on rats, 1 to 2 mgm. being sufficient to kill them in only a few hours (12).

METHODS. The rats were kept separately in cages, each of which contained a food cup and two graduated inverted 100 cc. bottles. The same two bottles were used in each cage throughout the experiment; and each bottle was marked and kept in the same corner of the cage, either on the right or on the left side. The fluid intake from the two bottles was recorded daily. For approximately 10 days both bottles were filled each day with distilled water. At the end of this time the intake from each bottle usually became fairly constant. Then one bottle was filled with a

subliminal concentration of a solution of phenylthiocarbamide, made with distilled water; and each day thereafter the concentration of the solution was increased in small steps. As the concentrations were increased, a point was finally reached at which the rats began to drink less phenylthiocarbamide solution and more water. The concentration at which they thus first indicated that they recognized the difference between phenylthiocarbamide solution and distilled water was taken as the taste threshold. The temperature of the solution varied with the room temperature which ranged from 24° to 26°C.

Of the 47 rats, 23 were males and 24 females. Records were kept of litter mates. The ages of the rats ranged from 70 to 152 days.

In the threshold determinations on human beings the subjects were seated at a table opposite the experimenter. Two one-ounce glasses, each containing about 5 cc. of distilled water, were placed in the subject's hands with instructions to sample the fluids and to describe the taste of each one. Then the two glasses were presented again, one containing distilled water, the other a subliminal concentration of phenylthiocarbamide. With each successive trial the concentration was increased in the same steps used for the rats. One glass always contained distilled water, the other a solution of phenylthiocarbamide. The glasses were indistinguishable, except for a small mark which was visible only to the experimenter. Their relative positions, to the right or to the left, were varied in an irregular order. A record was kept of the sensations described by the subjects for each concentration. When the subjects definitely stated that phenylthiocarbamide had a bitter taste or when it was found that they could not taste a 0.25 per cent solution, the test was discontinued. To make certain that no difference in temperature existed between them, the phenylthiocarbamide solution and the distilled water were checked at frequent intervals. They were kept at room temperature which ranged from 22° to 26°C.

Of the 261 human subjects, 139 were males and 122 were females. The ages varied from 17 to 50 years. Most of the subjects were students at the State Teachers College at Towson, Maryland, and at Loyola College; the others were members of the laboratory and clinical staffs at the Phipps Psychiatric Clinic.

RESULTS. *Taste thresholds of rats.* Figure 1 shows the record of a typical rat. The ordinates give fluid intake in cubic centimeters; the abscissas, time in days and concentrations of phenylthiocarbamide. During the preliminary period of seven days on two bottles of distilled water, the rat drank more from bottle 1 than from bottle 2. On the eighth day phenylthiocarbamide was added to bottle 1, from which the rat had been drinking more water. After 14 days, when the concentration of phenylthiocarbamide had reached 0.0003 per cent, the animal first began to drink less of the phenylthiocarbamide solution. This was taken as the taste

threshold. The rat completely refused all higher concentrations and drank only from the water bottle.

Figure 2A gives the frequency curves of taste thresholds for the 47 rats. The taste thresholds ranged from 0.00005 to 0.02 per cent. The maximum frequency fell at 0.0003 per cent. Two rats did not begin to drink less of the solution until the concentration reached 0.02 per cent.

Taste difference thresholds of human beings. Figure 2B shows the concentrations of phenylthiocarbamide solution at which each of the 261 human beings first stated that they detected a taste different from that of distilled water. They ranged from 0.000005 to 0.1 per cent. The maximum frequency fell at 0.0003 per cent, which is also the taste threshold for rats.

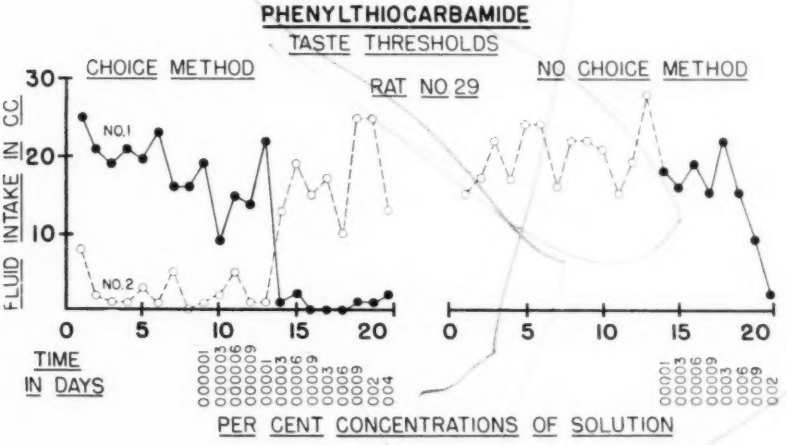

Fig. 1

When they first recognized a difference between the phenylthiocarbamide solution and distilled water, 125 of the 261 human subjects stated that it had a bitter taste; 136 subjects recognized a difference but did not report a bitter taste. Table 1 gives a list of the different taste expressions and the frequency of their use by these individuals.

Bitter taste thresholds of human beings. The concentrations of the phenylthiocarbamide solution at which the 261 human subjects first recognized a bitter taste ranged from 0.00001 to 0.2 per cent. See figure 2C. The maximum frequency, however, fell at 0.0003 per cent, which is the same concentration found for the difference thresholds for the same human subjects and for the taste thresholds of rats. At water saturation of phenylthiocarbamide (0.25 per cent) 13 individuals failed to recognize a bitter taste. These same individuals had, however, recognized a differ-

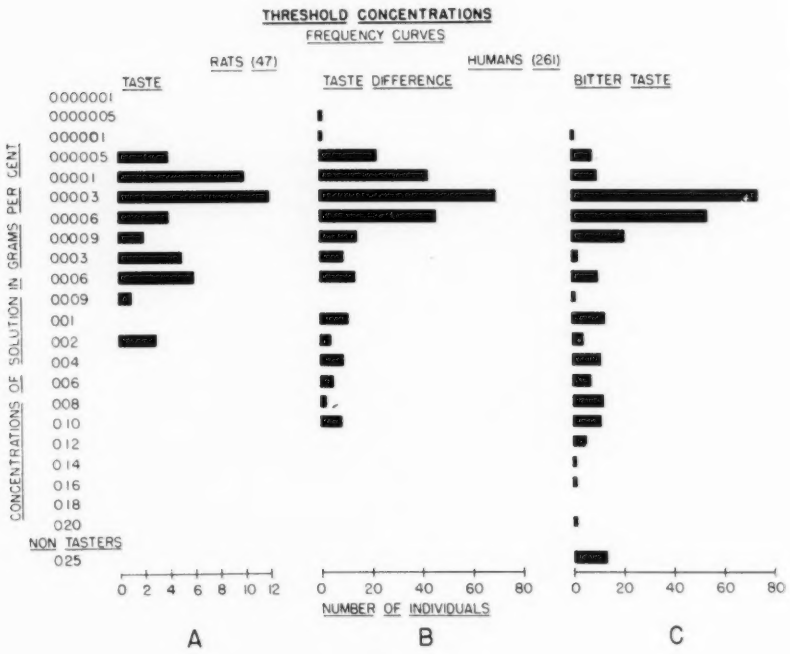

Fig. 2

TABLE 1

Expressions used to describe subliminal phenylthiocarbamide solutions as compared with distilled water and frequency of use

TASTE SENSATIONS	NUMBER OF SUBJECTS
Different	33
Stronger	18
Dry	11
Alum—Puckering	5
Burning	2
Sweet	15
Salty	5
Sour	17
Iron	2
Other sensations and combinations of the above	28
Total	136

ence in taste between the phenylthiocarbamide solution and distilled water. They objected to the taste of the phenylthiocarbamide even though it did not taste bitter to them. These results confirm the work of Salmon and

Blakeslee (5), who report a maximum frequency for bitter responses at 0.000312 per cent. In their study on 93 individuals, 3 per cent were non-tasters.

REMARKS. With the hope of simplifying the technique used on rats we attempted to determine the taste threshold with one bottle in place of two. For about 10 days this bottle was filled with distilled water, and daily records were taken to obtain a base line of fluid intake. Then the bottle was filled with a subliminal concentration of phenylthiocarbamide solution, and the concentrations were increased each day exactly as with the two-bottle technique. With this method the maximum frequency fell at 0.009 per cent, which was considerably above the threshold of 0.0003 per cent obtained with the two-bottle technique. The concentrations from 0.003 to 0.009 per cent included 41 of the 47 rats. At these concentrations the distastefulness of the solution must have overcome the thirst. Clearly, the taste threshold given by this method is far above the actual threshold. Figure 1 shows a typical record obtained with the one-bottle or no choice method.

Taste as a guide. The results of these experiments have demonstrated that most of the rats avoided the highly toxic phenylthiocarbamide even in very low concentrations. The question arises, then, as to whether rats could be made to take phenylthiocarbamide in lethal doses if it were offered in a solution with some substance for which they have a strong craving, or simply if it were mixed with the regular stock diet.

In one set of experiments 8 rats kept on the stock diet were offered a choice between plain water and a 20 per cent dextrose solution which contained 0.02 per cent of phenylthiocarbamide. Table 2 summarizes the results. For the 10 days before the phenylthiocarbamide was added the daily intake of the 20 per cent dextrose solution averaged 62 cc. When phenylthiocarbamide was added to the dextrose solution, the daily intake dropped sharply to 6.6 cc. for the first 24 hours. From their intake of this solution the rats received from 0.8 to 2.0 mgm. of phenylthiocarbamide per day—that is, amounts which fall at the lower limits of the lethal dose. Four of the rats died within 12 hours; the other 4 apparently did not drink enough of the dextrose solution to kill them. They gave no indications of any toxic effects.

This experiment was repeated on 8 rats with twice the amount of phenylthiocarbamide added to the dextrose solution (0.04 instead of 0.02 per cent). Here again 4 rats died and 4 showed no signs of toxic effects. The daily phenylthiocarbamide intake of the rats which lived ranged from 0.04 to 2.0 mgm. and of those that died, from 1.6 to 2.8 mgm. The determining factor in such an experiment would presumably be the amount of phenylthiocarbamide ingested at any one time.

In another experiment we offered 9 rats a choice between the regular

stock diet in one cup and the stock diet mixed with a certain amount of phenylthiocarbamide in a second cup. For 3 rats the food contained 500 mgm. of phenylthiocarbamide per 100 grams, or 0.5 per cent; for 3 rats the food contained 200 mgm. of phenylthiocarbamide per 100 grams, or 0.2 per cent; and for 3 rats it contained 100 mgm., or 0.1 per cent. Table 3 summarizes the results. The first 3 rats took from 2 to 3 grams of the phenylthiocarbamide food, thus ingesting 10 to 15 mgm. of phenylthio-

TABLE 2

RAT NUMBER	SEX	INTAKE OF DEXTROSE, 20 PER CENT, 10-DAY AVERAGE	INTAKE OF PTC, 0.02 PER CENT, AND DEXTROSE, 20 PER CENT, 1 DAY	INTAKE OF PTC	SURVIVAL TIME
		cc.	*cc.*	*mgm.*	
1	♂	62	10.0	2.0	12 hours or less
2	♂	70	8.0	1.6	12 hours—died
3	♂	44	6.0	1.2	12 hours—died
4	♂	74	6.0	1.2	12 hours—died
5	♂	59	9.0	1.8	Survived
6	♂	56	5.0	1.0	Survived
7	♂	60	5.0	1.0	Survived
8	♂	67	5.0	0.8	Survived
Average		62	6.6		
			PTC, 0.04 PER CENT, AND DEXTROSE, 20 PER CENT		
9	♂	65	7.0	2.8	12 hours—died
10	♂	60	5.0	2.0	12 hours—died
11	♂	53	4.0	1.6	12 hours—died
12	♂	53	4.0	1.6	12 hours—died
13	♂	65	5.0	2.0	Survived
14	♂	59	4.0	1.6	Survived
15	♂	49	2.0	0.8	Survived
16	♂	60	1.0	0.4	Survived
Average		57	4.5		

carbamide. They all died within a few hours. The second 3 rats ingested 1 to 2 grams of the phenylthiocarbamide food, thus getting 2 to 4 mgm. of phenylthiocarbamide. All 3 died. One of the last 3 rats ate 2 grams of the phenylthiocarbamide food and died; one rat ate 1 gram; the other did not eat a sufficient amount of food for weight determination. The latter 2 rats survived.

The high mortality of the rats that received the phenylthiocarbamide in their food may be explained by the high degree of insolubility of the

phenylthiocarbamide in powdered form. Undoubtedly the rats sampled the food in both cups. Since the phenylthiocarbamide is so highly insoluble, they must have eaten a lethal dose of the phenylthiocarbamide food before they recognized the bitter taste. The 50 per cent mortality of the rats which received phenylthiocarbamide in the dextrose solution may be explained partly by the possibility that the sweetness of the dextrose may have greatly reduced the bitter taste of the phenylthiocarbamide and partly by the possibility that the craving for the dextrose overcame the aversion to the phenylthiocarbamide. Since taste thresholds were not determined previously on these rats, it is possible that the animals that died may have been less sensitive to the bitter taste.

TABLE 3

RAT NUMBER	SEX	INTAKE		PTC IN FOOD	INTAKE OF PTC	SURVIVAL TIME
		Regular food	PTC food			
		grams	*grams*	*per cent*	*mgm.*	
17	♀	0	3	0.5	15	12 hours or less
18	♀	2	2	0.5	10	12 hours—died
19	♀	2	2	0.5	10	12 hours—died
20	♀	1	2	0.2	4	12 hours—died
21	♀	0	2	0.2	4	12 hours—died
22	♀	2	1	0.2	2	12 hours—died
23	♀	2	2	0.1	2	12 hours—died
24	♀	2	1	0.1	1	Survived
25	♀	2	0	0.1	0	Survived

Discussion. The results of these experiments bring further evidence for the close gustatory relationship of rats and human beings. We know now that rats and human beings have almost identically the same taste thresholds for common sugar, salt, and phenylthiocarbamide.

Since about 5 per cent of human beings scarcely taste the substance, or do not get its bitter taste at all, they very likely would take dangerously large amounts, even if the phenylthiocarbamide were offered in an aqueous solution. Human beings who are taste blind to phenylthiocarbamide and similar substances should be helped in their dietary selections and avoidances just as color blind individuals are helped by special signs and warnings.

SUMMARY

1. For the 47 rats the taste threshold concentrations ranged from 0.00005 to 0.02 per cent. The maximum frequency fell at 0.0003 per cent.

2. For the 261 human subjects the concentrations at which the phenylthiocarbamide solution first tasted different from distilled water ranged from 0.000005 to 0.1 per cent. The maximum frequency fell at 0.0003 per cent, at which concentration rats first indicated that they recognized a difference between the phenylthiocarbamide solution and distilled water.

3. The concentrations at which human subjects first recognized a bitter taste ranged from 0.00001 to 0.2 per cent. The maximum frequency for the 261 individuals again fell at 0.0003 per cent. Thirteen individuals never recognized a bitter taste even though they did recognize that the phenylthiocarbamide did not have the same taste as the distilled water. These results confirm those of Salmon and Blakeslee (5).

4. The concentration at which 95.5 per cent of the rats refused to take the phenylthiocarbamide solution fell below the lethal doses.

5. It was found that when phenylthiocarbamide was mixed with regular food most of the rats would eat enough to kill themselves. This may be explained by the fact that, due to the insolubility of phenylthiocarbamide, they swallow it before they taste it. When the phenylthiocarbamide was placed in a 20 per cent dextrose solution, 50 per cent of the rats drank sufficiently large amounts to kill themselves. The sweet flavor of the dextrose may have concealed the bitter taste of the phenylthiocarbamide.

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